Bioactive Metabolites from Halophilic Fungi

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ABSTRACT

Fungi are prolific sources of structurally novel and biologically active compounds. Over the past decades terrestrial fungi have been a major source of bioactive metabolites but little attention was paid to other habitats. Recently, increasing attention has been devoted to marine fungi as they too are a potential source of compounds for the development of new pharmaceutical agents.

In searching for bioactive metabolites from marine fungi, several extracts showed significant activity against the P388 murine leukaemia cell line. These strains were grown in different culture conditions to optimise bioactivity and the optimal conditions were chosen for scale up. Bioassay- and/or ^{1}H NMR-guided separation of active extracts led to the isolation of twenty two compounds. The structures of these compounds were elucidated using spectral analysis techniques especially mass spectrometry and NMR spectroscopy (^{1}H and ^{13}C NMR, COSY, NOESY, HSQC and CIGAR).

From a *Chaetomium* sp. (A34) the known compound chetomin was isolated. It exhibited very potent P388 activity with IC_{50} 11.2 ng/mL.

From an isolate of *Chaetomium globosum* (N60), three new cytochalasins named chaetoglobosins Q, R and S were isolated, together with nine previously characterised compounds: chaetoglobosins A, B, D and J; prochaetoglobosins I and II; chaetoviridins A and B, and ergosterol peroxide. In the assay against the P388 murine leukaemia cell line, most of these compounds exhibited significant activity (IC_{50} 1943-3988 ng/mL). In addition, some compounds also showed antimicrobial activities.

Examination of an isolate of *Phoma* resulted in the isolation of two simple

compounds: 2,5–dihydroxybenzoic acid and 2,5–dihydroxybenzyl alcohol. The former was inactive while the latter showed significant P388 activity (IC_{50} 3660 ng/mL).

Three compounds, identified as bikaverin, beauvericin and bassiatin, were isolated from the filtrate of *Fusarium* sp. (A145). In the P388 assay, these compounds exhibited strong, moderate and no activity respectively (IC_{50} 304, 4126 and > 12500 ng/mL, respectively).

An isolate of *Aspergillus fumigatus* (A151) was investigated for bioactive compounds. Culture broths incubated for different periods produced different metabolites. From the mycelium of a broth cultured for eleven weeks, one biologically active compound, xanthocillin-X dimethyl ether (IC_{50} 606 ng/mL for the P388 cell line), was obtained. From the filtrate of a four week culture, two known compounds, 12α–fumitremorgin C and fumagillin, and a novel compound, fumagiringillin, were isolated.

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ABBREVIATIONS

2D	two dimensional
Å	angstrom(s)
$[\alpha]_D^{20}$	specific rotation (at 589 nm at 20°C)
AIDS	acquired immune deficiency syndrome
APT	attached proton test (in NMR)
AT	acquisition time
ATA	alimentary toxic aleukia
BC	British Colombia
br	broad (spectral)
C°	degrees Celsius
C_{18}	octadecyl-phase (in column chromatography)
calcd.	calculated
CBA	carboxylic acid
CI	chemical ionization (mass spectrometry)
CIGAR	constant time inverse-detected gradient accordion rescaled long-range heteronuclear multiple bond correlation (in NMR)
cm	centimetre (s)
COSY	^{1}H-^{1}H correlation spectroscopy (in NMR)
CMA	cornmeal agar
CSA	Czapek solution agar
CYA	Czapek yeast extract agar
δ	chemical shift in parts per million downfield from tetramethylsilane
d	doublet (spectral)
DAS	diacetoxyscirpenol
DCM	dichloromethane

diam.	diameter
Diol	dialcohol-phase (in column chromatography)
DMSO	dimethyl sulfoxide
DON	deoxynivalenol
EtOAc	ethyl acetate
FVM	frozen vegetative mycelia
g	gram (s)
G25N	25% glycerol nitrate agar
hexanes	petroleum ether, boiling point 50-70 °C
HIV	human immunodeficiency virus
HMBC	heteronuclear multiple bond coherence (in NMR spectroscopy)
HPLC	high-performance liquid chromatography
HREIMS	high-resolution electron impact mass spectrometry
HRESMS	high-resolution electron spray mass spectrometry
HSQC	heteronuclear single quantum coherence (in NMR spectroscopy)
Hz	hertz
IR	infrared
J	coupling constant (in NMR spectroscopy)
L	litre (s)
λ_{max}	maximum wavelength
m	multiplet (spectral), metre (s), milli
MDR	multi-drug resistance
Me	methyl
MEA	malt extract agar
mg	milligram (s)
MHz	megahertz
MICs	minimum inhibitory concentrations
min	minute (s)
MS	mass spectrometry
MW	molecular weight
m/z	mass to charge ratio (in mass spectrometry)
ng	nanogram (s)

NGF	nerve growth factor
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
PAF	platelet activating factor
PDA	potato dextrose agar
PDB	potato dextrose broth
ppm	parts per million (in NMR spectroscopy)
q	quartet (spectral)
rpm	revolution per minute
RT	retention time
s	singlet (in NMR spectroscopy)
SDA	Sabouraud dextrose agar
S-MEA	seawater malt extract agar
S-PDA	seawater potato dextrose agar
t	triplet (in NMR spectroscopy)
T/C	ratio of the mean survival time of the treated animal (T) to that of the control animal (C)
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TMS	trimethylsilyl, tetramethylsilane
TOCSY	total correlation spectroscopy
UV	ultraviolet
YEA	yeast extract agar
μ	micro

Chapter 1

INTRODUCTION

1.1 Fungal Secondary Metabolites

Fungi are the second largest group of organisms in the world after the insects. It is estimated that there are one and a half million fungi in existence, but with just 5% of this total having being described in the literature.[1] Fungi are a very versatile and diverse group of eukaryotes. They have evolved to inhabit many different environments and have become one of the major sources in the search for bioactive compounds. A wide variety of biologically active fungal products, such as antibiotics, alkaloids, enzyme inhibitors and toxins are natural products referred to as secondary metabolites. For a long time secondary metabolites were thought to play no obvious role in the economy of the organisms. Although the evolutionary origin and the role of secondary metabolites for the producing organisms are still areas of vigorous debate, there is a growing consensus that secondary metabolism will benefit the organism and this benefit will usually reflect the nature of the metabolites themselves.[2]

Fungal secondary metabolites are relatively small molecules characterised, not only by their structural diversity,[3] but also by diversity of biological activity.[4] There is also a diversity in the species specificity of production, some being produced by a limited number of isolates of a single species whereas others may by produced by several species.

1.2 Important Pharmaceuticals Derived from Fungal Secondary Metabolites

The discovery of penicillin by Alexander Fleming in 1929 started the era of fungal-derived medicines. Penicillin, one of the most famous antibiotics, is a metabolite of *Penicillium notatum*. This "wonder drug" has saved literally millions of lives. Penicillin G (Figure 1.1), the original metabolite isolated, is still a "front line" antibiotic, in common use for some bacterial infections although the development of penicillin-resistance in several pathogenic bacteria now limits its effectiveness.

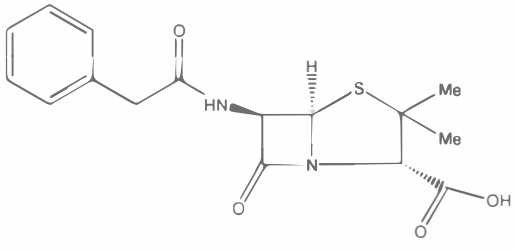


Figure 1.1 Structure of penicillin G

The phenomenal success of penicillin led to the search for other antibiotic-producing fungi. Griseofulvin and fusidic acid (Figure 1.2) are two examples of the successful search.

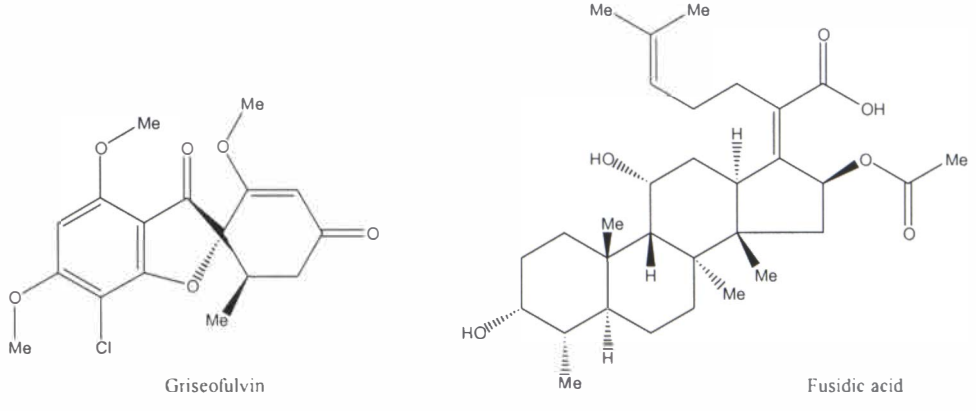


Figure 1.2 Structures of griseofulvin and fusidic acid

Griseofulvin is produced by *Penicillium griseofulvum* and related species[5] and is used to treat athlete's foot and related fungal infections of the skin.[6] Fusidic acid is a steroid produced by various Fungi Imperfecti. It is clinically used as an antibacterial

antibiotic and is known to inhibit protein synthesis.[7]

Cyclosporin A, mofetil and lovastatin are other well-known examples of fungal derived medicines. Cyclosporin A (Figure 1.3) was first discovered as an antifungal agent produced by *Tolypocladium inflatum*.[4] It was subsequently found to have excellent immunosuppressive activity and is the drug of choice to prevent the rejection of transplanted organs.[8]

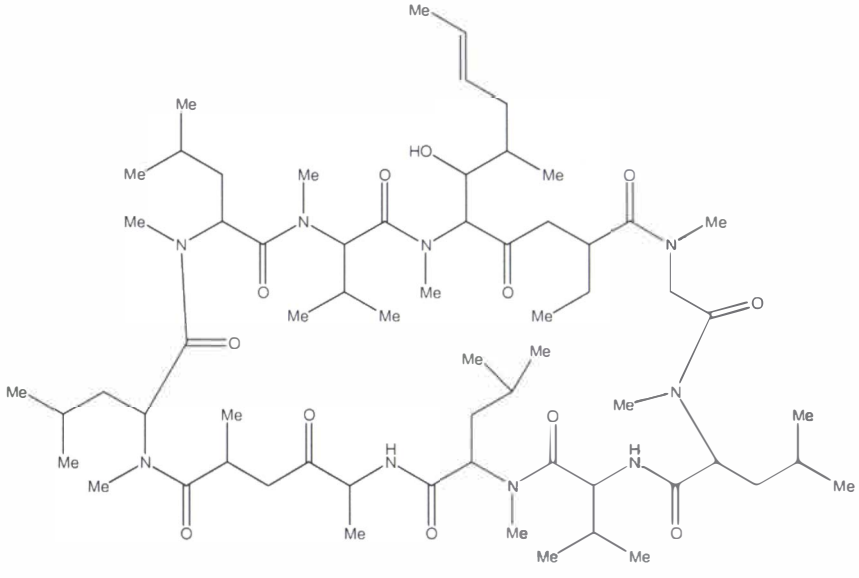


Figure 1.3 Structure of cyclosporin A

Mycophenolic acid is a fungal metabolite initially discovered from *Penicillium compactum* in 1896. In 1995, it was developed as an immunosuppressive agent and mofetil, a synthetic pro-drug derivative of this substance, is commercially available.[9]

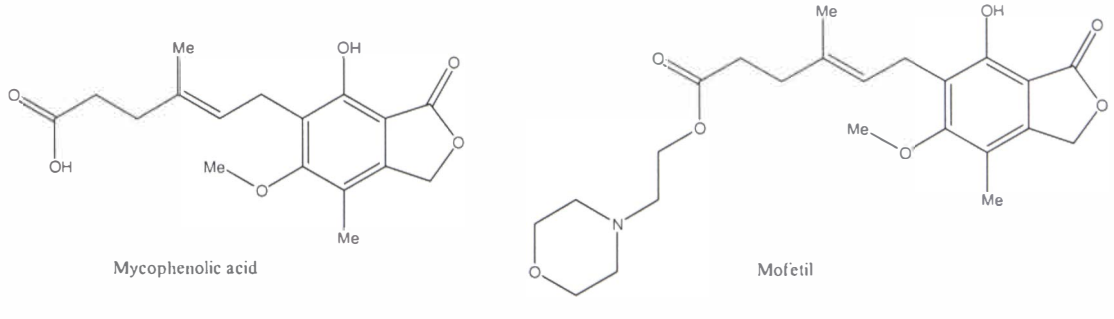


Figure 1.4 Structures of mycophenolic acid and mofetil

Lovastatin (MK-803, mevinolin) has been heralded as breakthrough-therapy for the treatment of atherosclerotic disease. It was initially isolated from *Aspergillus terreus*[10] and *Monascus rubber*.[11] Lovastatin is a highly potent inhibitor of HMG CoA reductase and used clinically to reduce cholesterol levels.[12]

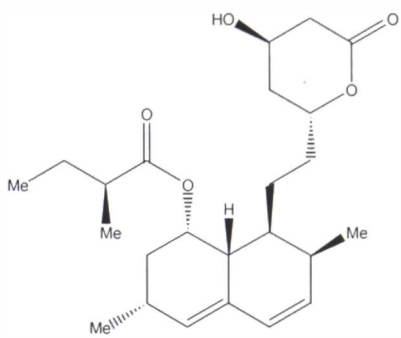


Figure 1.5 Structure of lovastatin

1.3 Marine Fungi as a Source of Novel, Bioactive Compounds

1.3.1 Definition of marine fungi

Marine fungi are not a taxonomic but an ecological group. They grow in oceans and ocean-associated estuarine habitats that contain brackish water including river mouths, tidal creeks and marshes, salinas, lagoons and the like. Marine fungi are widely distributed and have been isolated from various niches as diverse as seawater, sediments, algae, marine invertebrate and the gastrointestinal tract of marine fish. The definition of marine fungi has been described from different perspectives by different authors, with the criteria varying from the ability to grow in certain salt concentrations to the presence or absence of certain enzymes. However, Kohlmeyer and Kohlmeyer's definition has gained general acceptance.[13] In their proposal,[14] marine fungi are divided into two groups: *Obligate* marine fungi are those that grow and sporulate exclusively in marine or estuarine habitats; *facultative* marine fungi are those that originate from the land or freshwater and can grow and possibly sporulate in a marine environment. Fungi that are isolated from a marine environment but do not germinate in the natural marine habitat are not included in this definition.

It is estimated that there are about 1500 species of marine fungi in existence,

excluding those from lichens.[15] Among the described marine species, the most evolved groups (Ascomycotina and Deuteromycotina) predominate.[16] Just like their terrestrial counterparts, marine fungi also occur as parasites, saprophytes and symbionts colonising a wide range of marine animals and plants and on various plant debris in the sea.

As the aim of this study is to search for bioactive metabolites of halophilic fungi from the marine environment, any fungal strain isolated from the sea, estuaries, brackish waters and salt marshes that grew in a seawater medium was regarded as marine.

1.3.2 The potential for obtaining novel bioactive metabolites from marine fungi

Over the past 60 years, terrestrial fungi have been a major source of bioactive compounds but little attention was paid to other habitats. Decades of extensive research has made it increasingly difficult to isolate novel metabolites, hence new fungal sources are now under scrutiny. The oceans cover more than 70% of the Earth's surface, and represents a microbially-broad and diverse resource of huge dimensions but about which relatively little is known. According to Bernan's report, as of 1991, only 321 marine fungi had been described in the literature, compared to 69,000 terrestrial fungi.[17] Furthermore, the chemistry of marine fungi has also been widely neglected until recently with only some 168 metabolites from marine fungi reported to date.[16] Although terrestrial fungi will undoubtedly continue to be a productive source of new metabolites, increasing attention has been paid to marine fungi as they too are a potential source of bioactive substances for development of new pharmaceutical agents.

The initial interest in finding bioactive metabolites from marine fungi was also due, at least in part, to the suspicion that some metabolites obtained from marine algae and invertebrates could be produced by associated marine microorganisms. It has been established that marine macroorganisms such as algae, sponges, tunicates, molluscs and others have provided an enormous array of structurally unique, highly bioactive and biomedically utilitarian secondary metabolites. However, because of their low growth rate, low productivity of metabolites and complexity of chemical structures,

the supply of these compounds from nature is often limited, and the possibility of commercially viable syntheses is often eliminated. The fact that microorganisms, especially marine bacteria, were in some instances the true source of compounds isolated from marine macroorganisms has evoked wide interest in marine microorganisms. If a microbial origin is established for a compound of interest, it would in theory, be possible to obtain reasonable amounts of the valuable substance through large-scale fermentation. Recently, marine microorganisms especially bacteria and fungi, have gained prominence as a source of novel bioactive metabolites.

The metabolic and physiological capabilities of marine fungi that allow them to survive in their unique habitats also provide great potential for the production of metabolites not found in terrestrial environments. Fungi living in marine environments are expected to have different biosynthetic pathways or biosynthetic outcomes from their terrestrial counterparts. The differences are thought to result from the adaptation to the higher pressure and especially to the presence of salt. For example, the chemical constituents of the marine fungus *Trichoderma harzianum* grown in a fresh water medium differ significantly from those grown in seawater.[18] Another example is a marine strain of the genus *Aspergillus* from the Indo-Pacific sponge *Spirastrella vagabunda.*[19] This strain produces a chlorinated polyketide derivative in a seawater-derived medium but produces other metabolites in a freshwater culture medium.

In 1995, Pietra and co-workers[20] described their programme for evaluating the antimicrobial activity of marine and terrestrial fungi. Among the cultured 1500 marine and 1450 terrestrial fungal strains, more active marine strains (n=25) than terrestrial fungi (n=16) were found. Marine fungi produced more compounds with antifungal activity, while terrestrial fungal metabolites mainly exhibited activity against Gram-negative bacteria. In their study, only three new compounds were isolated from terrestrial strains, compared with 14 new compounds from marine strains.

Although comparatively little research has been directed toward the study of natural products from marine fungi, results from preliminary studies are encouraging. Initial investigations have shown that compounds isolated from marine fungi have

demonstrated antibacterial, antifungal, antitumour and other pharmacological activities. Therefore, marine fungi represent a relatively untapped, but promising resource for new bioactive compounds.

1.4 Bioactive Metabolites from Marine Fungi

Cephalosporin C, first described in the late forties, is the earliest report of a bioactive metabolite isolated from a marine fungus. It was produced by *Cephalosporium acremonium* isolated from seawater collected near a sewage outlet off the coast of Sardinia.[21] Cephalosporin C (Figure 1.6) is a β-lactam with a mode of action similar to that of penicillin, but with less allergenicity. To date, it is still the most important metabolite of marine fungi in clinical use as an antibiotic.

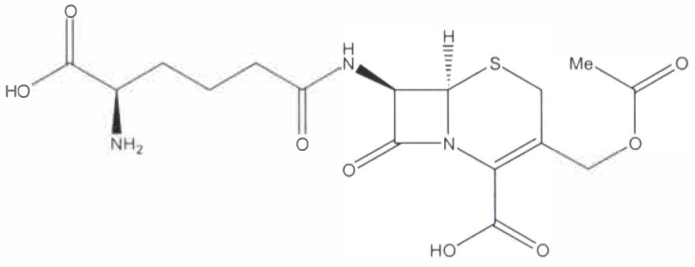


Figure 1.6 Structure of cephalosporin C

Although cephalosporin C was discovered in 1940's, an analysis of the literature shows that few novel bioactive metabolites from marine fungi were reported before the 1980's. From the 1990's, there was a rapid progress in this field and a number of reviews have since been dedicated to this topic.[17,22-25] Two extensive reviews of metabolites from marine fungi were published in 1998[13] and 2000.[16] The most recent bioactive metabolites from marine fungi are listed in Table 1.1. In this chapter, rather than repeating the conclusions of a comprehensive review, a few structurally interesting and highly active marine fungal metabolites will be examined.

Table 1.1 Bioactive metabolites recently isolated from marine fungi

Producing Strain	Compound	Activity	Reference
Acremonium sp.	Oxepinamides A [a] Fumiquinazolines H-I [b]	Antiinflammatory [a] Antifungal [b]	26
Acremonium striatisporum	Virescenosides O, P, Q	Cytotoxic	27
Acremonium striatisporum	Virescenosides M, N	Cytotoxic	28
Aigialus parvus	Aigialomycin D	Antimalarial	29
Ascochyta salicorniae	Ascosalipyrrolidinone A	Antiplasmodial Antimicrobial Enzyme inhibition	30
Aspergillus versicolor	Anthcolorins A - H	Cytotoxic	31
Aspergillus parasiticus	Parasitenone	Radical scavenging	32
Aspergillus sp.	Avrainvillamide	Cytotoxic	33
Cladosporium herbarum	Pandangolide 3, 4	Antimicrobial	34
Curvularia lunata	Lunatin	Antimicrobial	35
Drechslera dematioidea	Drechslerines E, G	Antiplasmodial	36
Emericella variecolor	Varitriol [c] Varixanthone [d]	Cytotoxic [c] Antimicrobial [d]	37
Eutypa sp.	Eutypoid A	Enzyme inhibition	38
Fusarium heterosporum	Mangicols A - G	Cytotoxic Anti-inflammatory	39
Fusarium sp.	*N*-Methylsansalvamide	Cytotoxic	40
Fusarium sp.	Sansalvamide	Cytotoxic	41
Gliocladium roseum	Roselipins	DGAT inhibitory Antimicrobial	42
Halorosellinia oceanica	Halorosellinic acid	Antimalarial Antimycobacterial	43
Leptosphaeria sp.	Leptosins M, M_1, N, N_1	Cytotoxic	44

Producing Strain	Compound	Activity	Reference
Myrothecium roridum	12,13-deoxyroridin E	Cytotoxic	[45]
Paecilomyces sp.	Paecilospirone Phomopsidin	Inhibition of microtubule proteins	[46]
Penicillium sp.	Farnesylhydroquinone	Radical scavenging	[47]
Penicillium sp.	Penochalasins D - H	Cytotoxic	[48]
Penicillium sp.	Penostatins H- O	Cytotoxic	[49]
Penicillium sp.	Sculezonones A, B	Enzyme inhibition	[50, 51]
Penicillium sp.	11,11'-dideoxyverticillin A 11'-deoxyverticillin A	Cytotoxic	[52]
Pestalotia sp.	Pestalone	Cytotoxic Antimicrobial	[53]
Scytalidium sp.	Halovir	Antiviral	[22, 54]
Ulocladium botrytis	Ulocladol	Enzyme inhibition	[55]
Unidentified fungus	Chlorogentisylquinone	Enzyme inhibition	[56]
Unidentified fungus	Dictyonamide A	Enzyme inhibition	[57]
Unidentified fungus	Spiroxins	Cytotoxic Antibiotic	[58]
Xylaria sp.	Xyloketal A	Enzyme inhibition	[59]

Investigations into marine fungi led Gloer's group to isolate an obligate marine strain of *Leptosphaeria obiones* from the coastal marsh grass *Spartina alterniflora* where the fungus is commonly found in the lower portion of the plant. Fermentation of this fungus resulted in the isolation of a new polyketide, obionin A.[60] Obionin A (Figure 1.7) inhibited the binding of dopamine D-1-selective ligands to membranes found in the central nervous system (IC_{50} of 2.5 μg/mL).

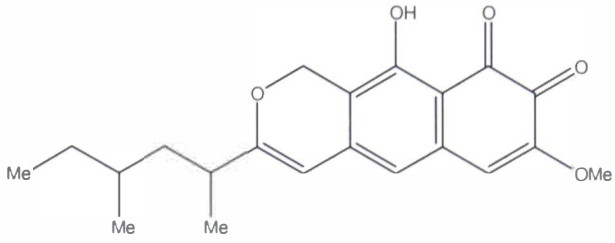


Figure 1.7 Structure of obionin A

In 1997, Varoglu *et al.* reported the isolation of a marine strain of *Aspergillus niger* from the sponge *Hyrtios* sp. A unique, asymmetric diketopiperazine dimer, asperazine (Figure 1.8), was isolated from the saltwater culture of the strain. Asperazine displayed an unusual profile of cytotoxicity and selective anti-leukaemic activity at 50 μg/disk in a soft agar disk-diffusion assay. The compound showed no antimicrobial activity.[61]

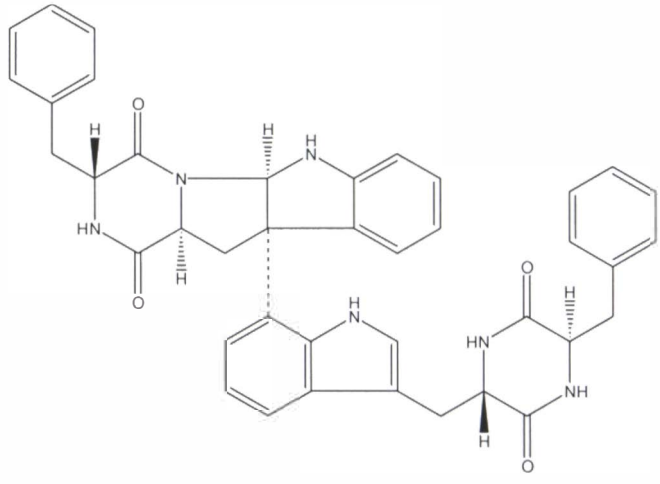


Figure 1.8 Structure of asperazine

Fellutamides A and B, two lipophilic tripeptides, were produced by *Penicillium fellutanum*, isolated from the gastrointestinal tract of the marine fish *Apogon endekataenia*.[62] Fellutamides A and B (Figure 1.9) showed potent cytotoxicity against murine leukaemia P388 and L1210 cells, and human epidermoid carcinoma KB cells *in vitro* (IC_{50} 0.1 - 0.8 μg/mL). Fellutamide A was also found to be a potent agent in stimulating the synthesis of nerve growth factor (NGF) and enhancing secretion of this factor *in vitro* in L-M cells, rat brain cells and rat glial cells. This compound

showed a different mode of action from a known NGF inducer, epinephrine.[63]

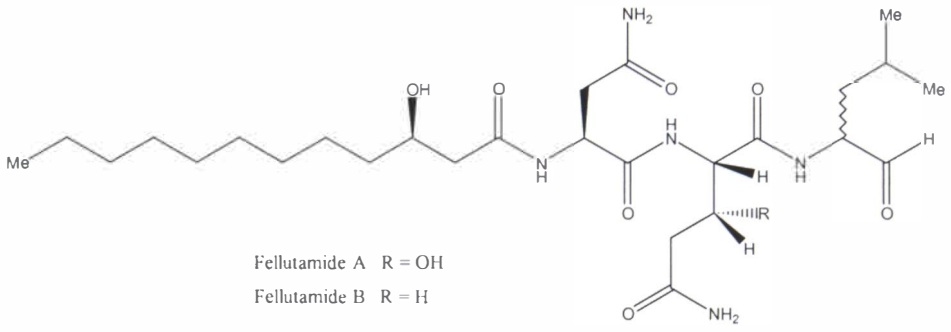


Figure 1.9 Structures of fellutamides

An obligate marine isolate of *Leptosphaeria*, separated from the marine brown alga *Sargassum tortile* is a rich source of diketopiperazine dimers. Nineteen dimeric epipolysulfanyldioxopiperazines, leptosins A – F,[64] the G group (G, G1, G2), H – J,[65, 66] the K group (K, K1, K2),[67] the M group (M, M1) and the N group (N, N1)[44] have been isolated and characterized by Numata and co-workers. Leptosins displayed significant P388 *in vitro* cytotoxicity (IC_{50} 1 - 4 μg/mL for most of the compounds). More importantly, leptosins A and C (Figure 1.10) displayed potent *in vivo* antitumour activity against Sarcoma-180 ascites (T/C 260 % and 293 %) at doses of 0.5 mg/kg and 0.25 mg/kg, respectively.

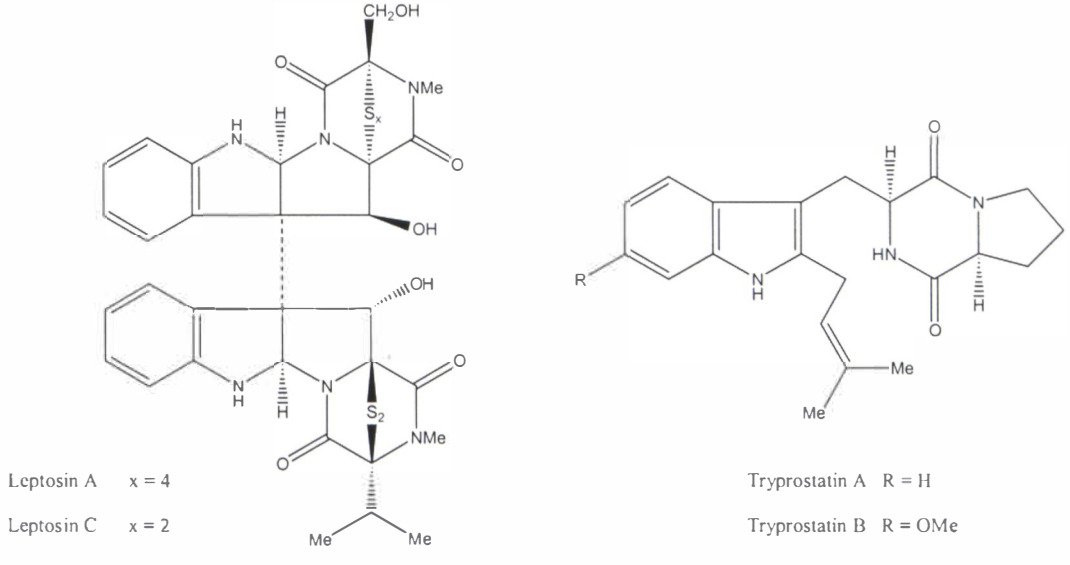


Figure 1.10 Structures of leptosins and tryprostatins

Tryprostatins A and B (Figure 1.10) are tryptophan-based fungal metabolites that inhibit the mammalian cell cycle.[68] The producing culture, *Aspergillus fumigatus* BM939, was isolated from a sediment sample collected at the depth of 760 m at the mouth of the Oi river in Japan. Tryprostatins are structurally related to fumitremorgins, the metabolites of the terrestrial counterpart of *A. fumigatus*. Tryprostatins A and B complete inhibited cell cycle progression of tsFT210 cells in the G2/M phase at a final concentration of 50 and 12.5 μg/mL, respectively.

In an effort to discover new antitumour compounds from microorganisms inhabiting the marine environment, Numata's group[69] reported three cytotoxic cytochalasans, penochalasins A-C (Figure 1.11) from a marine *Penicillium* sp. The producing strain was originally separated from the marine alga *Enteromorpha intestinalis*. Subsequent studies of this fungus led to the isolation of penochalasins D-H.[48] Penochalasins A-D constitute a novel class of cytochalasins, with a macrocyclic ring including a pyrrole moiety. In the *in vitro* assay for activity against cultured P388 cells, all the compounds exhibited significant cytotoxicity (ED_{50} 0.3-3.2 μg/mL).

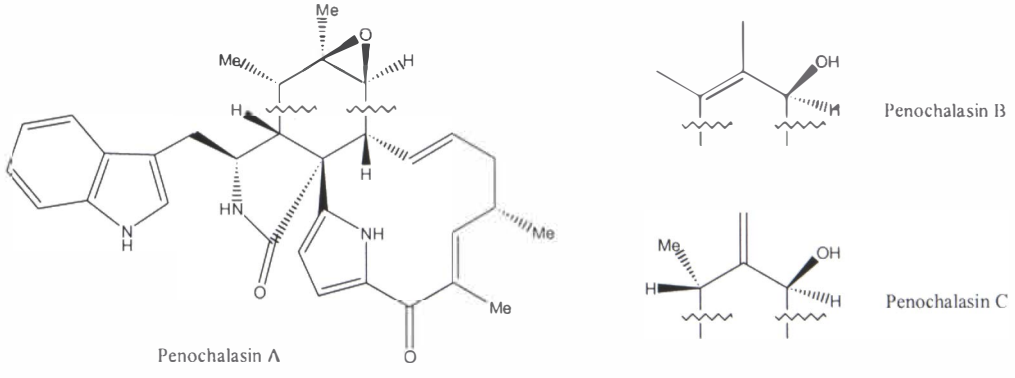


Figure 1.11 Structures of penochalasins

The same *Penicillium* sp. cultured on different media led to the isolation of four new compounds, penostatins A-D.[70] Subsequently, penostatins F-O[49, 71] were isolated from the same source. It is interesting to note that this fungus produces some pairs of stereoisomers in which all the asymmetric centres except for C-5 have the opposite absolute configurations. Penostatins are terpenoids (Figure 1.12) and all the compounds except for penostatin D exhibited significant cytotoxicity against cultured P388 cells (IC_{50} 0.5 – 1.4 μg/mL).

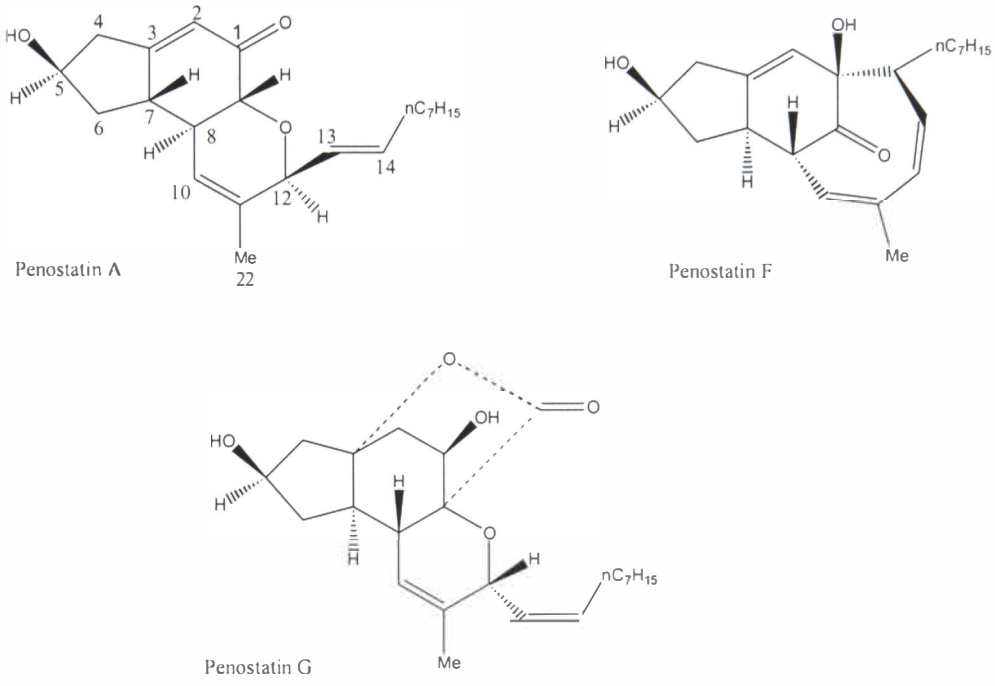


Figure 1.12 Structures of penostatins

Schlingmann and co-workers at Wyeth-Ayerst isolated an antifungal agent 15G256 γ from the marine fungus *Hypoxylon oceanicum*. 15G256 γ (Figure 1.13) belongs to a new class of lipodepsipeptides and inhibits fungal cell wall biosynthesis.[72] The compound possesses broad-spectrum antifungal activity and is able to provide good disease control against a wide variety of phytopathogenic fungi when used at high concentrations (in the range of 500 μg/mL, in foliar sprays). *In vitro* testing showed moderate activity against a series of human fungal pathogens (MICs: 2-16 μg/mL).[73]

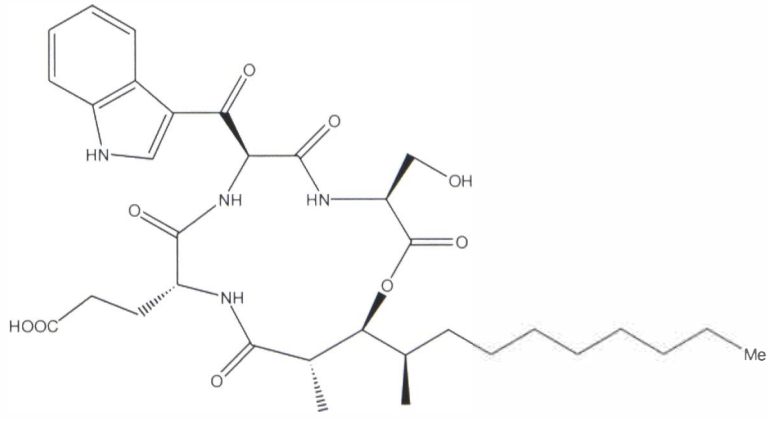


Figure 1.13 Structure of compound 15G256 γ

In their programme to search for biologically active secondary metabolites from marine fungi, Fenical's group reported the isolation of a new class of sesterterpenes - neomangicols A-C[74] and mangicols A-G.[39] The producing strain, tentatively identified as *Fusarium heterosporum*, was isolated from mangrove drift-wood. In *in vitro* assays, most of the compounds showed weak to modest cytotoxicity against a series of cancer cell lines. However, neomangicol B (Figure 1.14) showed potent antibacterial activity against *Bacillus subtilis*, and mangicols A and B (Figure 1.14) exhibited significant anti-inflammatory activity.

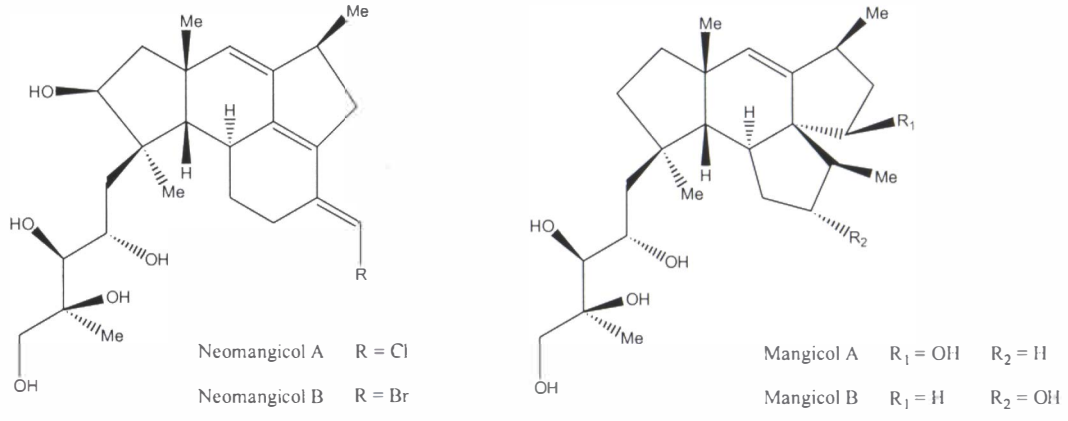


Figure 1.14 Structures of neomangicols and mangicols

Recently, the same group[22,54] reported the isolation and characterization of three novel peptides, halovirs A-C. These compounds are produced by a marine *Scytalidium* sp., separated from a sample of seagrass, *Halodule wrightii*. Halovir A (Figure 1.15) exhibits potent antiviral activity against the *Herpes simplex* virus 1 (IC_{50} 1.1 μg/mL). Furthermore, 0.6 M halovir A completely inhibits HSV-I-induced cell lysis after an exposure of 30 minutes. The compound thus may be of potential use as a lead for a topical antiviral agent.

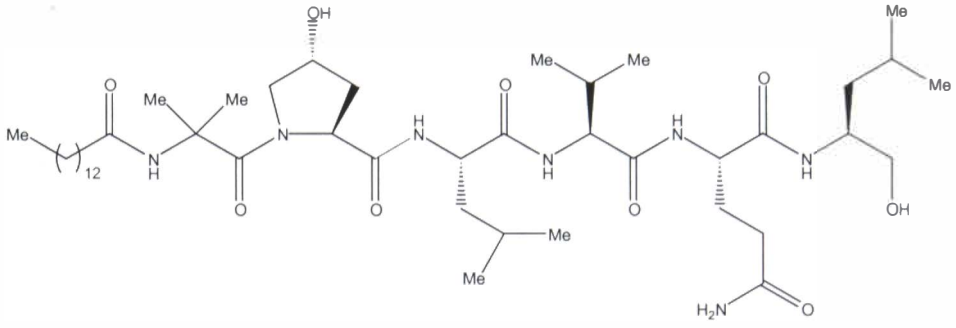


Figure 1.15 Structure of halovir A

1.5 Discussion

The secondary metabolites reported to date from marine fungi represent diverse structural classes that include polyketides, alkaloids, terpenes and compounds of mixed biosynthetic origin. An interesting characteristic of the alkaloids found to date is the high frequency of occurrence of complex diketopiperazines. These occur as monomers or dimers and quite often possess sulfanyl groups of different chain lengths.

It is noteworthy that many of the metabolites of marine fungi are similar to or identical to those found from terrestrial fungi. One reason suggested for this observation is that some of the species studied only occasionally inhabit the marine environment. Another reason is that the same or similar secondary metabolites could be produced by diverse microbial taxa. However, marine fungi can also produce metabolites with unique carbon skeletons. Marine fungi generally follow the broad outlines of terrestrial fungal biosynthesis, but there are some examples indicating that marine conditions can modify the nature of the metabolites produced leading to the production of novel compounds not found in the terrestrial environment.

The majority of biologically active metabolites from marine fungi reported to date show cytotoxic and/or antimicrobial activity. This is mainly due to the fact that most isolations have been carried out with the assistance of bio-assay guided separation since the 1990's. Since the assays are generally selected based upon the source of the research funding or availability through collaborators, cytotoxic and antimicrobial assays are widely used in most laboratories. With increasing interest in this field, it is

believed that metabolites with more diverse biomedical activities will soon be explored.

The number of secondary metabolites isolated from marine fungi is still small but is rapidly increasing. There is speculation that highly adapted marine fungi such as symbionts, or those inhabiting extreme environments such as the deep sea, offer greater opportunity for the production of novel metabolites. Being an unexplored field, exploration of marine-based fungi may afford unexpected new metabolites endowed with interesting pharmacological properties.

1.6 Work Done at the University Of Canterbury

The Marine Chemistry Group at the University of Canterbury initiated a programme for exploring bioactive metabolites from fungi in 1998. The majority of the fungal strains have been isolated from the New Zealand marine and terrestrial environment such as marine algae, marine and freshwater sediments, rainforests, endemic plants and insects. Some strains have been isolated from other niches such as Himalayan soils, Antarctic soils and marine sediments. The extracts from the culture of these strains have been tested for cytotoxicity, antimicrobial and antiviral activity *in vitro*. Promising extracts are evaluated further by chemical screening before starting a bioassay-guided separation. To date, a series of biologically active metabolites, including some novel compounds, have been isolated from these strains.

1.7 The Aim of This Study

The aim of the research is to investigate isolates of fungi separated from saline environments which have initially been shown to possess biological activity. The metabolite(s) responsible for the activity will be isolated using bioassay-guided separation and the structures of pure compounds will be elucidated using mass spectrometry, ^{1}H NMR, ^{13}C NMR and a series of 2D NMR spectroscopic techniques.

Chapter 2

CULTURE AND SCREENING PROGRAMME

2.1 Introduction

In the search for biologically active compounds from natural resources, the Marine Chemistry Group at the University of Canterbury isolated a large number of fungal strains from the marine environment. These strains were cultured on a small-scale (250 mL) and tested for cytotoxicity against the P388 murine leukaemia cell line, and for antimicrobial activity against a range of bacteria and fungi and antiviral activity against *Herpes simplex* and *Polio* viruses (see section **7.1.3** , **Chapter 7**). Active strains were chosen for further investigation to isolate the bioactive compounds. In this study, eight strains which showed significant cytotoxicity were screened and grown in different culture conditions to optimise bioactivity. In addition, other strains were also surveyed for P388 activity. Finally, five fungi were targeted for further investigation and it is these fungi that form the basis for this research.

Before initiating a bioassay-guided isolation, the extracts from those fungi of interest were usually examined by a strategy called "chemical screening". Chemical screening is a method used to assess the properties of an extract and is used routinely at the University of Canterbury (see section **2.5**).

2.2 Selection of Culture Conditions to Optimise Bioactivity

Biological activity of fungal extracts is mediated by the production of one or more compounds (secondary metabolites). It is generally known that the production of these metabolites can be influenced by many factors such as the composition of medium, temperature, aeration, culture time, etc. For the eight bioactive fungal strains isolated in our primary screen, a survey of different media and agitation conditions was undertaken to optimise bioactivity. The isolates were first cultured on different solid media to test their growth. They were then cultured in liquid media to obtain extracts and to assay for P388 activity. Finally, the optimal culture conditions were chosen for scale up in order to obtain the pure compounds.

The fungal isolates used for optimizing activity were as follows: A145 (*Fusarium* sp.); A151 (*Aspergillus* sp.); K31 (*Phoma* sp.); A34, A116, A127, A128 and A266 (*Chaetomium* spp.).

2.2.1 Growth of isolates on solid media

The selected isolates were tested for growth rate on six different nutrient agar media: potato dextrose agar (PDA), seawater potato dextrose agar (S-PDA), malt extract agar (MEA), yeast extract agar (YEA), Sabouraud dextrose agar (SDA) and Czapek solution agar (CSA) (see section **7.1.2 , Chapter 7**). Although all isolates were from a marine environment, none was obligately marine and all grew in the absence of seawater on normal media.

Process for measuring growth rate: A mycelial disc (4 mm in diam.) was cut from the growing margin of a PDA colony of the appropriate isolate. The disc was placed at the centre of an 85 mm Petri dish containing 20 mL of test medium. The dishes were then incubated at 25 °C in darkness. Measurements were made at known intervals along two diameters of the colony at right angles to each other. The diameter of a 24-hour colony was used as a base to calculate the rate of growth over the following few days until the colony reached the edge of the plate. Results are shown in Table 2.1.

Table 2.1 The growth rate of marine fungi on different agar media

Code	Genus	Growth Rate on Different Media (mm/day)					
		PDA	S-PDA	SDA	MEA	YEA	CSA
A145	*Fusarium*	9.2	9.2	11.3	10.8	11.7	11.2
A151	*Aspergillus*	9.6	11.2	12.2	12.2	9.2	11.4
K31	*Phoma*	9.1	8.0	10.8	9.4	9.0	7.0
A34	*Chaetomium*	6.0	6.1	7.0	8.5	10.5	7.6
A116	*Chaetomium*	5.2	3.8	7.0	7.7	9.5	6.5
A127	*Chaetomium*	4.7	4.4	7.3	8.1	10.4	6.1
A128	*Chaetomium*	4.9	5.6	6.3	8.0	8.4	5.2
A266	*Chaetomium*	5.2	5.2	6.4	7.7	7.4	4.5

2.2.2 Growth of isolates in liquid media

Each isolate was cultured in four different liquid media previously shown to be beneficial for secondary metabolite production: potato dextrose broth, malt extract broth and yeast extract broth at half normal strength (1/2 PDB, 1/2 MEB and 1/2 YEB, respectively). In addition, a seawater medium (S-XXB) was also prepared for each fungus based on its growth and sporulation on solid medium. For each medium, both shake (rotary shaker at 180 rpm) and static conditions were used to determine if agitation would improve the production of bioactivity.

Each fungus was grown in two 250 mL Erlenmeyer flasks each containing 125 mL of appropriate medium. Following a 28 day fermentation period at 26 °C, the mycelium and broth were separated by filtration and extracted with EtOAc respectively. The extracts were then combined and made up to 10 mg/mL of methanol solution for P388 assay. The results are summarized in Table 2.2 (for strains A145, A151 and K31) and Table 2.3 (for strains A34, A116, A127, A128 and A266).

For each fungus, the optimal culture condition was selected based mainly on the P388 activity. Where P388 activities were close, the yield of extract was used as an additional selection criterion.

Table 2.2 Yield and P388 activity of isolates cultured in different broths

Code	Genus	Medium	Condition	Extract Yield (mg)	P388 Activity (IC_{50} ng/mL)
A145	*Fusarium*	1/2 PDB	Shake	142.9	5417
		1/2 PDB	Static	115.0	19460
		1/2 MEB	Shake	23.6	2619
		1/2 MEB	Static	41.3	39092
		1/2 YEB	Shake	49.3	5741
		1/2 YEB	Static	50.4	22504
		1/2 S-PDB	Shake	85.6	4684
		1/2 S-PDB	Static	71.2	11378
A151	*Aspergillus*	1/2 PDB	Shake	104.5	3712
		1/2 PDB	Static	39.3	1598
		1/2 MEB	Shake	65.9	17840
		1/2 MEB	Static	68.3	3606
		1/2 YEB	Shake	18.1	24554
		1/2 YEB	Static	35.1	2200
		1/2 S-PDB	Shake	96.3	8624
		1/2 S-PDB	Static	35.1	12955
K31	*Phoma*	1/2 PDB	Shake	54.1	5910
		1/2 PDB	Static	126.5	4964
		1/2 MEB	Shake	40.7	14135
		1/2 MEB	Static	61.0	6639
		1/2 YEB	Shake	30.5	96249
		1/2 YEB	Static	43.3	14552
		1/2 S-MEB	Shake	22.3	43912
		1/2 S-MEB	Static	74.3	11873

Table 2.2 indicates that the P388 activity of fungus A145 (*Fusarium* sp.) was largely influenced by agitation rather than by medium type. Activities in the four different media were generally similar while cultures in shaken conditions were more active than those in static conditions. Since a good extract yield was afforded in a culture of ½ PDB (shake), this condition was chosen for the large scale fermentation (more than 4 litres).

Most cultures of the fungus A151 (*Aspergillus* sp.) showed similar moderate P388 activity except for ½ MEB (shaken), ½ YEB (shaken) and ½ S-PDB (static) which exhibited very weak activity. Since a considerable amount of extract was yielded from the culture of ½ PDB (shake), this condition was chosen for the scale up.

For the fungus K31 (*Phoma* sp.), only cultures in ½ PDB (shaken and static) and ½ MEB (static) showed moderate P388 activity. It is clear that ½ PDB in static conditions was best for this fungus.

Table 2.3 Yield and P388 activity of *Chaetomium* isolates cultured in different broths

Code	Medium	Condition	Extract Yield (mg)	P388 Activity (IC_{50} ng/mL)
A34	1/2 PDB	Shake	34.0	114586
A128	1/2 PDB	Shake	65.9	>125000
A266	1/2 PDB	Shake	54.6	>125000
A34	1/2 PDB	Static	41.8	30034
A128	1/2 PDB	Static	47.9	21233
A266	1/2 PDB	Static	43.9	3210
A34	1/2 MEB	Shake	33.5	<975
A128	1/2 MEB	Shake	24.8	<975
A266	1/2 MEB	Shake	25.9	<975
A34	1/2 MEB	Static	18.2	<975
A128	1/2 MEB	Static	26.7	<975
A266	1/2 MEB	Static	23.2	<975
A34	1/2 YEB	Shake	54.0	85685
A128	1/2 YEB	Shake	25.4	17835
A266	1/2 YEB	Shake	21.0	83239
A34	1/2 YEB	Static	74.1	7677
A128	1/2 YEB	Static	32.8	4550
A266	1/2 YEB	Static	31.4	3712
A34	1/2 S-MEB	Shake	46.8	19.4
A116	1/2 S-MEB	Shake	40.1	28.4
A127	1/2 S-MEB	Shake	41.5	27.4
A128	1/2 S-MEB	Shake	47.9	88.0
A266	1/2 S-MEB	Shake	43.2	153.0
A34	1/2 S-MEB	Static	16.1	50
A128	1/2 S-MEB	Static	27.8	93
A266	1/2 S-MEB	Static	21.9	105

The P388 activity for strains cultured in ½ S-MEB were measured at 0.02 mg/mL

Table 2.3 indicates that the medium had significant influence on P388 activity of *Chaetomium* isolates while agitation did not improve the bioactivity. Most cultures in ½ PDB and ½ YEB showed very weak or no activity while cultures in ½ MEB and ½ S-MEB exhibited very significant activity. The presence of seawater in any medium was irrelevant to bioactivity but increased the extract yield.

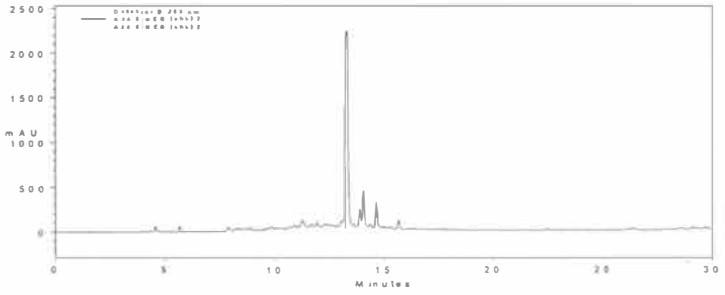
The consistent bioactivity of strains A34, A128 and A266 in different media and agitation conditions suggested that these strains might belong to one species and produce the same bioactive compounds. The observation of growth rate, maturation of the ascomata, size and shape of ascomata and ascospores revealed that these morphological characteristics were identical in all the five strains (A34, A116, A127, A128 and A266). HPLC analysis as shown in section **2.3** indicates that these strains showed exactly the same HPLC profiles.

To screen for the most active strains, A116 and A127 were cultured on ½ S-MEB in agitation since this condition was found to give best activity and extract yield. By comparing the P388 activity and extract yield, strain A34, which showed the strongest activity against the P388 murine leukaemia cell line (IC_{50} 19.4 ng/mL), was chosen for large scale fermentation.

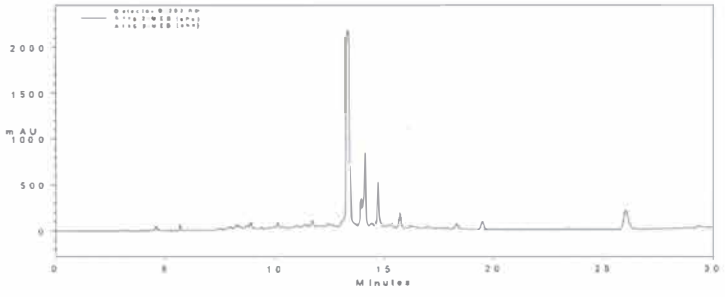
2.3 HPLC of Extracts from the *Chaetomium* Isolates

2.3.1 HPLC of different strains cultured on the same medium

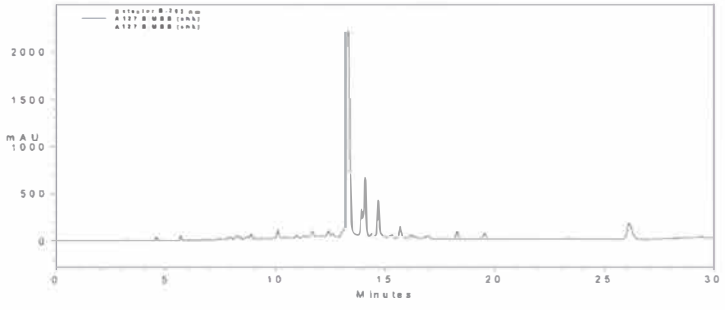
The 5 strains of *Chaetomium* (code: A34, A116, A127, A128 and A266), as mentioned above, belonged to one species and exhibited consistent bioactivity in the P388 assay. To examine the composition of their secondary metabolites, extracts of ½ S-MEB broths (shake conditions) of these 5 strains were subjected to high performance liquid chromatography (HPLC), eluted with a ACN/H_2O gradient (standard conditions, see section **7.1.4 , Chapter 7**). Identical HPLC profiles (UV detector at 203 nm) are shown in Figure 2.1, indicating these strains produce the same secondary metabolites under the test conditions.



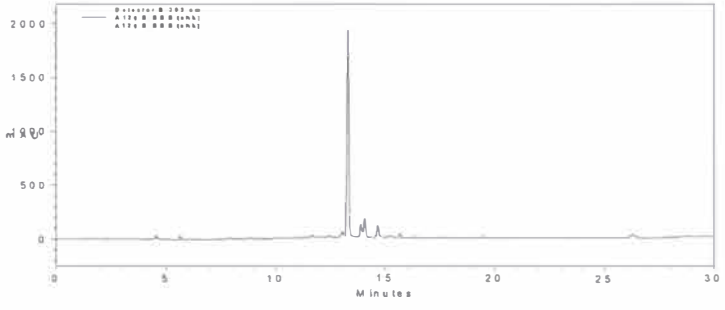
A34 Extract



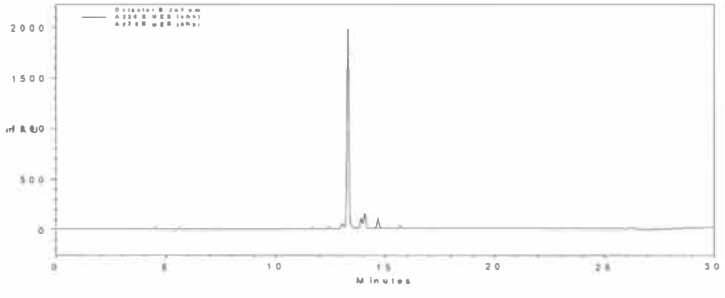
A116 Extract



A127 Extract



A128 Extract

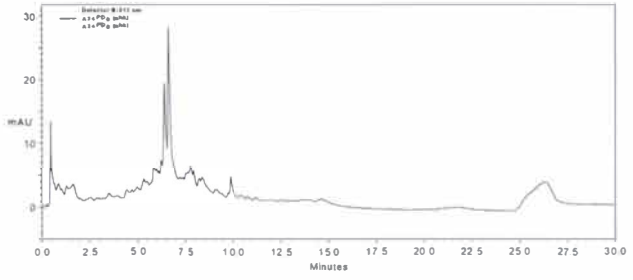


A226 Extract

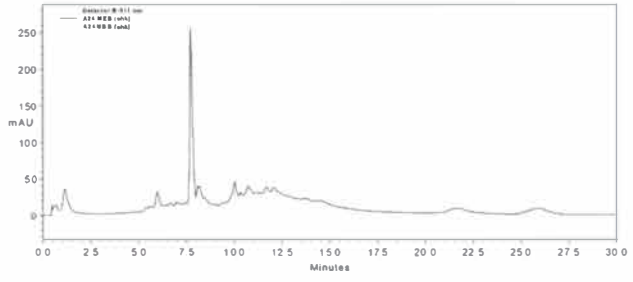
Figure 2.1 HPLC profiles of 5 strains of *Chaetomium*

2.3.2 HPLC of extracts from strain A34 (*Chaetomium sp.*)

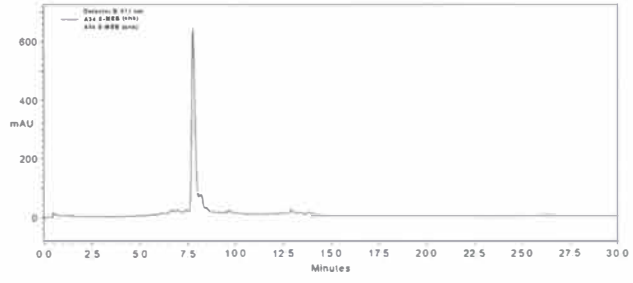
Results as shown in Table 2.3 indicate that growth medium has a considerable effect on P388 activity of the 5 *Chaetomium* strains and differences in bioactivity suggest the production of different metabolites. To confirm this assumption, one strain (A34) was cultured in ½ PDB, ½ MEB, ½ S-MEB and ½ YEB (all shake cultures) and the extracts were tested by HPLC (standard conditions). The profiles are shown in Figure 2.2 (UV detector at 311 nm).



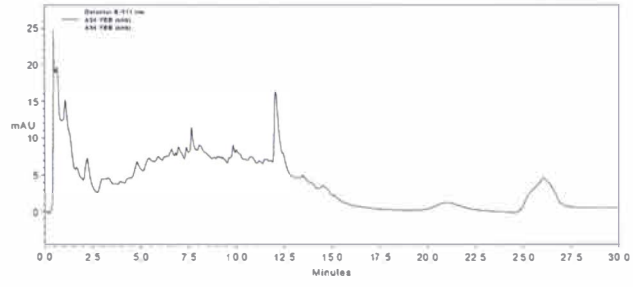
1/2 PDB Extract



1/2 MEB Extract



1/2 S-MEB Extract



1/2 YEB Extract

Figure 2.2 HPLC profiles of A34 extracts from 4 different media

It was noted that the chemical constituents of strain A34 grown in ½ MEB and ½ S-MEB were similar and differed significantly from those grown in ½ PDB and ½ YEB. Seawater did not induce the production of different metabolites but increased the concentration of bioactive compound(s). This result is consistent with the P388 activity observed in Table 2.3.

2.4 Screening of Other Fungi for P388 Activity

Four more fungal strains, one of *Chaetomium* and three of *Phoma*, were also surveyed for their activity against the P388 cell line. Initial cultures were grown on PDA for 7 days. Mycelial discs were then transferred to liquid media. Following a 28-day fermentation period at 26 °C, the mycelium and broth were separated by filtration and extracted with EtOAc respectively. The extracts of both parts were assayed for P388 activity and the results are presented in Table 2.4.

Table 2.4 Screening of fungal strains for P388 activity

Fungus	Culture Condition	Extract Yield		P388 activity (IC_{50} ng/mL)	
		Mycelium	Filtrate	Mycelium	Filtrate
N60 (*Chaetomium*)	1/2 MEB (2 L), shake	234 mg	294 mg	1942	2325
T881 (*Phoma*)	1/2 PDB (1 L), static	515 mg	8 mg	>125000	>12500
T882 (*Phoma*)	1/2 PDB (1 L), static	498 mg	33 mg	95475	26213
T883 (*Phoma*)	1/2 PDB (1 L), shake	172 mg	5 mg	55676	16727

Three isolates of *Phoma* (T881, T882 and T883) showed very weak or no activity against the P388 murine leukaemia cell line while the isolate of *Chaetomium* (N60) exhibited good activity. Since the morphological characteristics and the HPLC profiles of N60 were totally different from those of *Chaetomium* A34, it was chosen for further investigation.

In conclusion, the following fungi were chosen for detailed study of their bioactive metabolites: A145 (*Fusarium* sp.), A151 (*Aspergillus* sp.), K31 (*Phoma* sp.), A34 (*Chaetomium* sp.) and N60 (*Chaetomium* sp.).

2.5 Chemical Screening

Chemical screening, the chemical equivalent of biological screening, is a procedure developed by the Marine Chemistry Group at the University of Canterbury. The aim of the method is to achieve a rapid evaluation of physical, chromatographic and stability properties of interesting components and the dereplication of the known compounds before starting a formal separation.

2.5.1 Chemical screening (1) – solid phase extraction

In chemical screening (1), a crude extract of a fungus was passed through three cartridges of different sorbent phases. Generally, C_{18}, CBA and LH-20 cartridges were selected, and they separated components by polarity, charge and molecular size respectively. Three or four fractions were taken from each cartridge, and these along with the standard were tested to ascertain where the bioactivity had been distributed. Table 2.5 summarizes the results for five targeted fungi. It is observed that most active components in the tested fungi were small to medium size molecules, non-cationic and with medium polarity (except for K31 in which the most active components were very polar).

Table 2.5 Chemical screening results of five fungi

Sample		P388 Activity (IC_{50})									
Code	Genus	C_{18} cartridge			CBA cartridge			LH-20 cartridge			
		1	2	3	1	2	3	1	2	3	4
A145	*Fusarium*		++	+	++	+			+	++	+
A151	*Aspergillus*	+	++		+				+	++	
A34	*Chaetomium*		++	++	++	++	++		+	++	
K31	*Phoma*	++	+		++					++	
N60	*Chaetomium*		++		++					++	

N.B. ++ very active; + active

2.5.2 Chemical screening (2) - HPLC microtitre plate collection

In chemical screening (2), the most active fraction(s) from each cartridge and the standard were analysed by HPLC under identical conditions (C_{18} column, ACN/H_2O gradient, standard conditions). The peaks in common on these HPLC profiles were likely to be the compound(s) responsible for the bioactivity observed. A search on Antibase[75] using the UV data of active peak(s) resulted in a few cases where a unique answer could be obtained, however, in most circumstances tens to hundreds of hits were found. To establish a more meaningful search, the HPLC eluents of the most active fraction from the LH-20 cartridge in chemical screening (1) were collected into a microtitre plate (total 89 fractions) which was called the master plate. Two daughter plates were generated from it and assayed for P388 activity. Once the wells containing the bioactivity were located, ESMS of the corresponding active wells in the master plate were carried out. For example, in the case of A34 (*Chaetomium* sp.), chetomin was identified from the active microtitre plate wells by searching in Antibase using MW(MH^+ 711), UV data and fungus source. Another example can be seen for N60 (*Chaetomium* sp.) where ESMS of the components in the active wells established compound(s) with MW of 590 Daltons. A searching in Antibase came up with three components: chaetoglobosins A, B and D.

2.6 Discussion

Results in the experiments for optimising the P388 activity of isolated marine fungi revealed that bioactivity (or the secondary metabolites) can be significantly influenced by the composition of the medium (isolate A34) and agitation conditions (isolate A145). There appears to be no rules about which factor will lead to the desired results. As for industrial fermentations, optimal conditions are achieved largely by trial and error.

Seawater was expected to have some effects on the production of secondary metabolites, but no significant changes were observed for the tested fungi.

As indicated in section 2.5 , physical, chromatographic and stability properties of an extract can be rapidly obtained by chemical screening. With the help of this

information, the dereplication, isolation of active compounds and structural elucidation can be carried out more efficiently.

Chapter 3

CHAETOMIUM SPP. (A34 AND N60)

3.1 Introduction

3.1.1 General

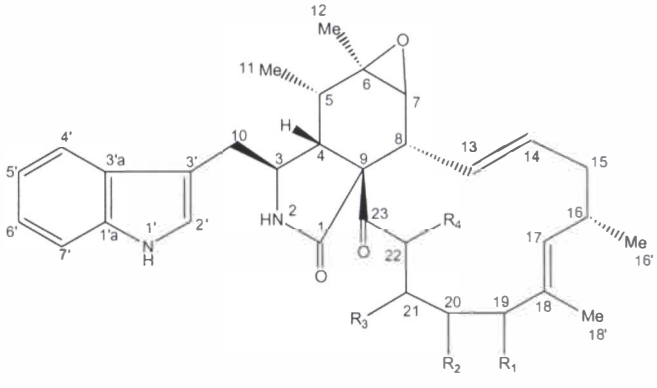
Chaetomium is one of the largest genera of saprophytic Ascomycetes and more than 300 species have been described. *Chaetomium* species are characterised by their ascomata (perithecia), being covered with numerous long hairs which give the genus its name (Greek *Chaite* = long hair, mane). The ascomata are produced superficially and are connected to the substrate by rhizoidal hyphae. They usually have an opening (ostiole) through which the ascospores are released. The ascospores are aseptate, often limoniform, brown or grey-olivaceous with a germ pore at one or both ends and often pushed out of the ostiole in a cirrhus.[76] *Chaetomium* species are notable as producers of cellulases and so commonly occur on wood and paper products.

3.1.2 Bioactive metabolites from Chaetomium species

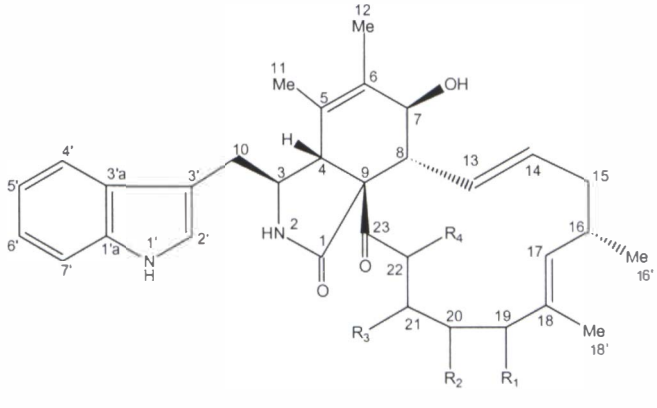
Species of *Chaetomium* occur widely in nature, and certain *Chaetomium* species are known to produce biologically active metabolites. Chaetoglobosins, epipolythiodioxopiperazines, chaetochromins and cochliodinols are among the most important bioactive compounds isolated from *Chaetomium* species and are further discussed here.

Chaetoglobosins

Chaetoglobosins are a family of secondary metabolites belonging to the cytochalasins. The name "cytochalasin" originates from the Greek word *cytos* (cell) and *chalasis* (relaxation) and refers to the cytological effects of the metabolites. The cytochalasins are remarkable in their biological activity including the inhibition of movement and cytoplasmic cleavage of mammalian cells. Much of the biological activity can be accounted for by an interaction with the common target protein actin. These biological activities are unprecedented by any other chemical compounds.[77] Chaetoglobosins contain an indolyl group (Figure 3.1) in place of the phenyl group found in other cytochalasans.[78]



	R_1	R_2	R_3	R_4
Chaetoglobosin A	OH	=O	$\Delta^{21,22}$	
Chaetoglobosin C	=O	=O	H	H
Chaetoglobosin F	=O	OH	H	H



	R_1	R_2	R_3	R_4
Chaetoglobosin B	OH	=O	$\Delta^{21,22}$	
Chaetoglobosin E	=O	OH	H	H
Chaetoglobosin G	=O	=O	H	H

Figure 3.1 Structures of some chaetoglobosins

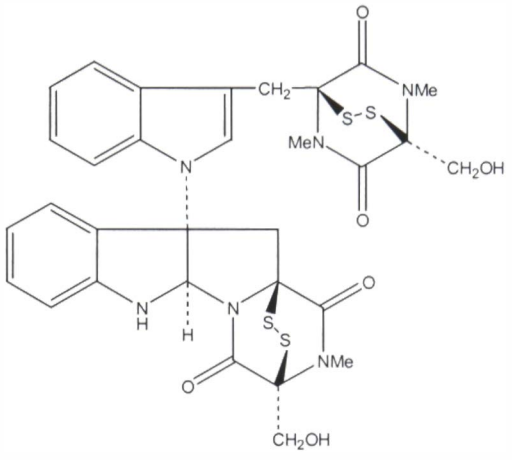
So far more than 20 chaetoglobosins have been reported and most of them are produced by *Chaetomium* species. Chaetoglobosins A, B, C, D, E, F, G and J, isolated from *Chaetomium globosum,*[79,80] show cytotoxicity against HeLa cells with an IC_{50}

value of 3.2 – 10 μg/mL (IC_{50} of chaetoglobosin C is 20 μg/mL).[81] Most chaetoglobosins can induce polynucleation in HeLa cells. Apart from cytotoxicity, some chaetoglobosins (A, C and O) also show phytotoxicity against alfalfa seedlings.[82] Chaetoglobosin A (Figure 3.1) is the most widely studied metabolite in the chaetoglobosin family and has been reported to exhibit strong cytotoxicity against other human cancer cell lines,[83] antibacterial activity against *Helicobacter pylori* and *Staphylococcus aureus*[84] and antifungal activity towards a series of plant pathogens.[85]

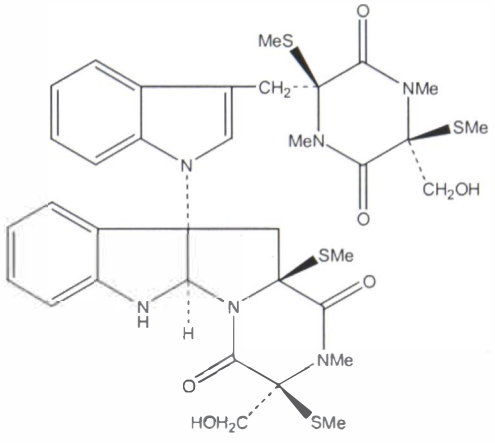
Epipolythiodioxopiperazines

Epipolythiodioxopiperazines such as gliotoxin and sporidesmins occur widely as fungal secondary metabolites and possess antiviral and antimicrobial properties as well as high mammalian toxicities. *Chaetomium* is one of the main genera which produce such compounds. Compounds belonging to this class include chetomin, dethio–tetra(methylthio)chetomin, chetocin, chetocins B and C, chetracin A and 11α, 11'α-dihydroxychaeocin (melinacidin IV) (Figure 3.2). These have been isolated from a series of *Chaetomium* species[86-89] and most exhibit quite remarkable cytotoxicity against HeLa cells (with IC_{50} values of 0.02 – 0.04 μg/mL).[88] These compounds also show remarkable activity towards Gram-positive bacteria while exhibiting only weak or no activity towards Gram-negative bacteria.[88,90] Chetomin, the first epipolythiodioxopiperazine isolated from *Chaetomium* species, also shows strong antifungal activity against *Pythium ultimum*. This activity is comparable to the fungicide metalaxyl and is 5-10 times higher than gliotoxin.[91]

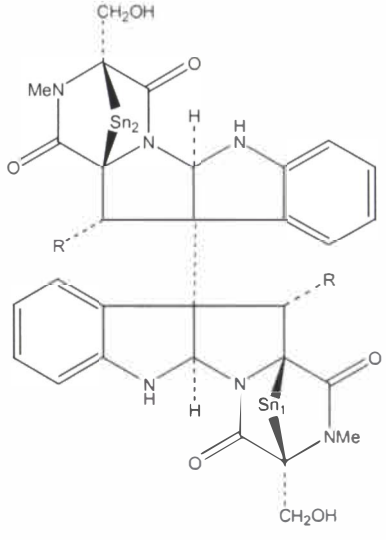
In studies of the structure-activity relationships of epipolythiodioxopiperazines isolated from *Chaetomium* species and their derivatives, it was found that the number of sulphur atoms influences the antimicrobial and cytotoxic activities.[88,92] Compounds with trisulfide bridges showed the strongest activities, followed by compounds with disulfide or tetrasulfide bridges, whereas the derivatives without bridges show much less or no activity.



Chetomin



Dethio-tetra(methylthio)chetomin



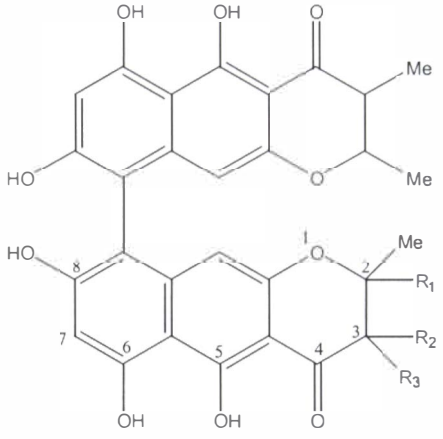
	R	n_1	n_2
Chaetocin	H	2	2
Chaetocin B	H	3	2
Chaetocin C	H	3	3
Chetracin A	OH	4	4
Melinacidin IV	OH	2	2

Figure 3.2 Structures of some epipolythiodioxopiperazines

Chaetochromins

Chaetochromins are bis (naphtho-γ-pyrone) derivatives (Figure 3.3). Chaetochromin A was first isolated from *Chaetomium thielavioideum*[93] and chaetochromins B, C and D were isolated from *C. gracile* thereafter.[94] Although chaetochromins A-D exhibit strong cytotoxicity in assay against KB cells, and chaetochromin A is effective in the treatment of MX-1 breast xenograft, M5076 ovarian carcinoma, and P388 lymphocytic leukaemia in mice, development of these compounds as antitumour agents was abandoned due to their toxicity and marginal activity.[95] On the other hand, according to a patent by Merck & Co., certain chaetochromins are useful in the

inhibition of HIV (human immunodeficiency virus) integrase, for the prevention or treatment of infection by HIV and the treatment of AIDS (acquired immune deficiency syndrome).[96]

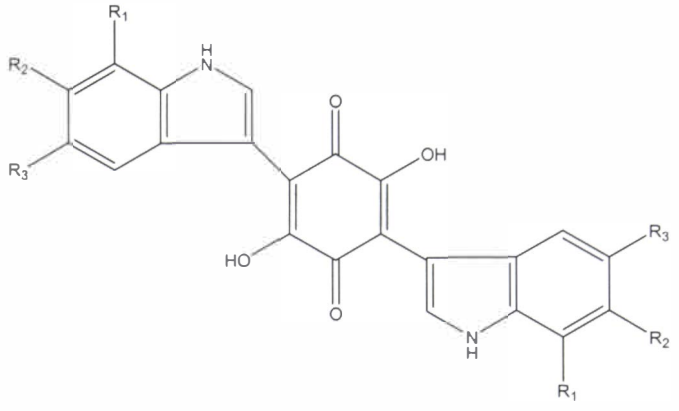


	R_1	R_2	R_3
Chaetochromin A	H	H	CH_3
Chaetochromin B	H	CH_3	H
Chaetochromin C	H	H	H
Chaetochromin D	$\Delta^{2,3}$		CH_3

Figure 3.3 Structures of chaetochromins A-D

Cochliodinols

Cochliodinols are purple pigments with a bis(3-indolyl)-dihydroxybenzoquinone nucleus in their structures. Cochliodinol (Figure 3.4) was isolated from *Chaetomium cochliodes* and *C. globosum*[97] while two related compounds, isocochliodinol and neocochliodinol (Figure 3.4) were isolated from *C. murorum* and *C. amygdalisporum* respectively.[98] Cochliodinol shows remarkable antibacterial and antifungal activity.[99] Brewer and colleagues[100,101] compared the antibacterial and antifungal activity of a number of naturally occurring 2,5-dihydroxy-1,4-benzoquinones against a range of bacteria belonging to 8 genera and 12 species of fungi belonging to 6 diverse genera. Among the tested compounds, cochliodinol exhibited relatively high antibacterial activity and was more active against Gram-positive bacteria. In the antifungal assay, cochliodinol was the most active compound and showed remarkable activity in both inhibiting vegetative growth and spore germination of fungi.



	R_1	R_2	R_3
Cochliodinol	H	H	$CH_2CH=CHC(CH_3)_2$
Isocochliodinol	H	$CH_2CH=CHC(CH_3)_2$	H
Neocochliodinol	$CH_2CH=CHC(CH_3)_2$	H	H

Figure 3.4 Structures of chochliodinols

Part I *Chaetomium* sp. (A34)

3.2 Culturing and Isolation of *Chaetomium* sp. (A34)

3.2.1 Culturing and extraction

The fungal strain CANU A34 was isolated from saline mud in western Australia and identified as belonging to the genus *Chaetomium* (Figure 3.5). The isolate was grown on PDA for 7 days at 25 °C. The mycelial disks (8 mm in diameter) were then cut from the agar and transferred into eleven litres of half-strength seawater malt extract broth. The broth was incubated at 26 °C for 30 days under agitating conditions. The culture was filtered through Celite 545 under suction to separate mycelium and filtrate. The mycelium and filtrate were then extracted separately with EtOAc. The extracts were evaporated under reduced pressure to give 284.2 mg of filtrate extract and 2242.9 mg of mycelial extract. In cytotoxicity assays against the P388 cell line, the mycelial extract showed stronger activity (IC_{50} 25 ng/mL) than the filtrate extract (IC_{50} 234 ng/mL).

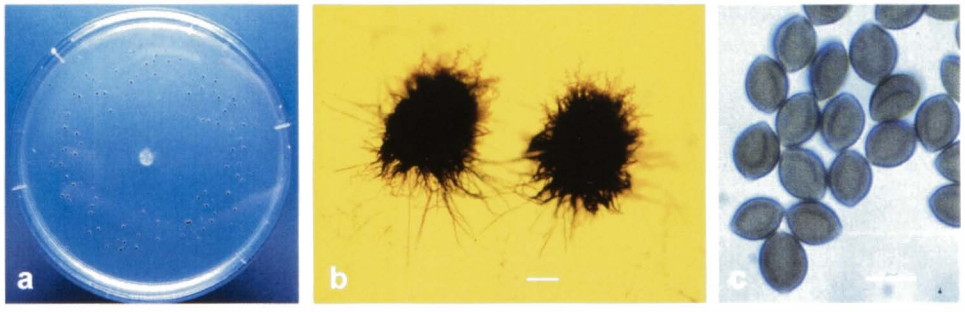
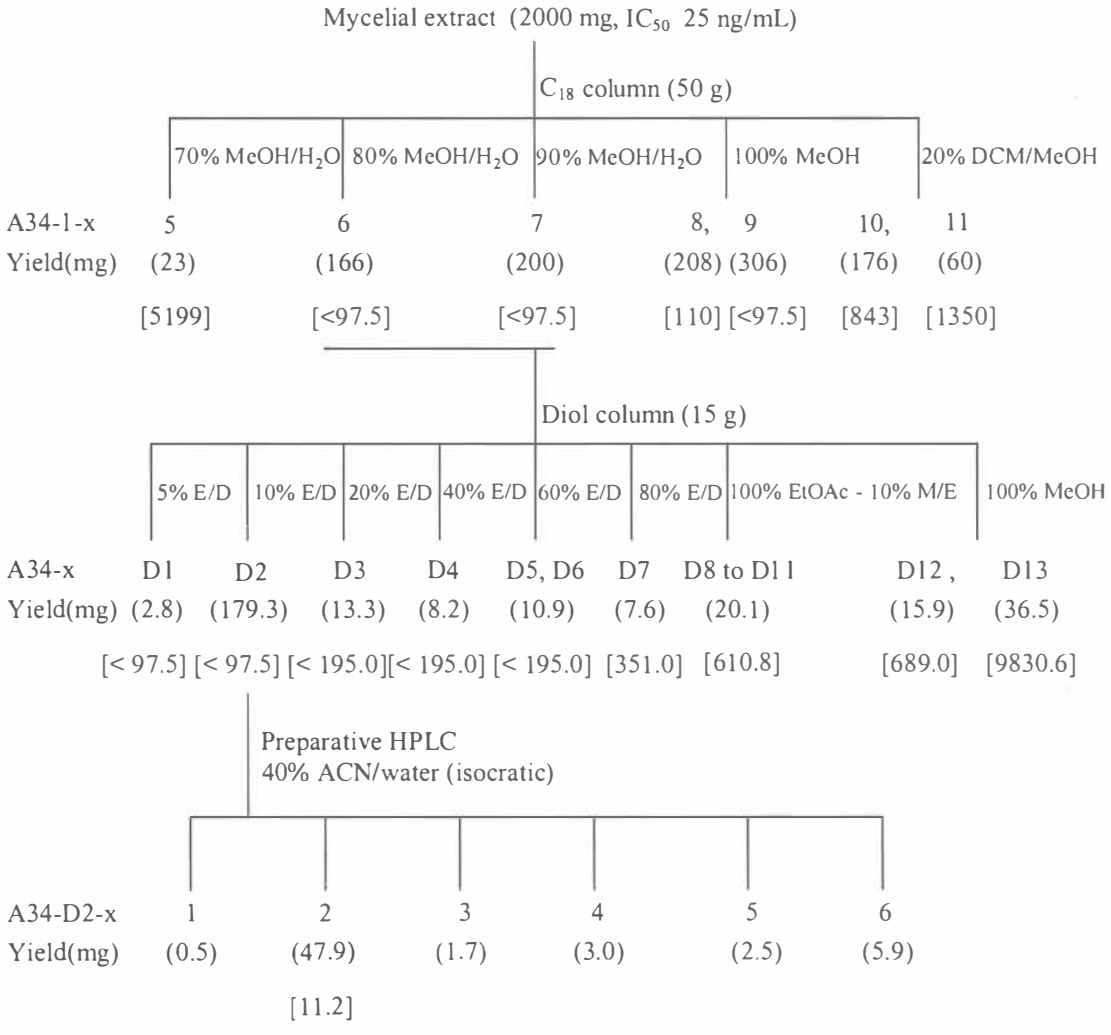


Figure 3.5 *Chaetomium* sp. (A34) (a) colony on CMA (26 days, 25 °C) (b) ascomata (c) ascospores; Bars: (b) = 100 µm; (c) = 10 µm

3.2.2 Chromatography

An outline of the chromatography is shown in **Scheme 3.1**.

Scheme 3.1



[ ]: P388 activity (IC_{50} = ng/mL)
E/D: EtOAc/DCM
M/E: MeOH/EtOAc

The mycelial extract (2000.0 mg) was chromatographed by a reverse phase (C_{18}, 50 g) column, eluted with a stepped gradient from 10% MeOH/H_2O to MeOH then to CH_2Cl_2. Fifteen fractions were collected and most showed activity against the P388

murine leukaemia cell line (IC_{50} < 97.5 – 5199 ng/mL). The activity focused on the fractions A34-1-6, 7, 8 and 9 and decreased gradually in adjacent fractions (Scheme 3.1). High performance liquid chromatography (HPLC) revealed fractions A34-1-6, 7, 8, 9 and 10 had very close HPLC profiles, and all contained a major peak at RT 15.00 minutes with several minor peaks varying only in ratios. However, fractions A34-1-5 and A34-1-11 had different HPLC profiles with a small peak at RT 15.00 minutes. This suggested that the compound at RT 15.00 minutes in the HPLC profiles represented the major activity observed and that the wide distribution of the activity was due to the poor solubility of the active compound(s). The ^1H NMR spectra also indicated that the major compound in fractions A34-1-6, 7, 8, 9 and 10 was the same. A34-1-11 was predominantly fatty acids whilst A34-1-5 was a complex mixture.

Fractions A34-1-6 and A34-1-7 were combined (totally 366 mg) and loaded onto a normal phase (Diol, 15 g) column. The column was eluted with a stepped gradient system from 5% EtOAc/CH_2Cl_2 to EtOAc and then to MeOH. Thirteen fractions were collected and combination of the fractions made after analysis by TLC. In the P388 assay, all the fractions were active (Scheme 3.1). Fractions A34-D1 and D2 were the most active fractions and the activity decreased gradually thereafter. In the HPLC profiles, A34-D2 was relatively pure, showing a major peak at RT 15.00 minutes and several very small minor peaks. The major peak of A34-D2 was also observed as a major peak on the fractions A34-D1, D3 to D6, and as a minor peak in fractions A34-D7 to D13. Again, the major peak was responsible for the P388 activity and the poor solubility of the compound led to the wide distribution of the activity.

A34-D2 (65.0 mg) was further purified using semi-preparative HPLC (C_{18} column, reverse phase), eluted with 40% ACN/H_2O isocratically. Six fractions were collected and fraction A34-D2-2 was afforded as a pure compound (47.9 mg). This compound was the major active compound observed previously and showed very significant activity against the P388 murine leukaemia cell line (IC_{50} 11.2 ng/mL). Fractions A34-D2-3, 4 and 5 were mixtures in which A34-D2-2 was still a major compound while two or three related compounds were the minor ones. To obtain these minor compounds, A34-D2-3 and A34-D2-4 were further purified using chromatography on a LH-20 column and reverse phase HPLC respectively. However, due to poor

solubility of the major compound (A34-D2-2) and the small amount of related compounds in the mixture, no pure compounds were isolated.

Since fraction A34-D2 showed a very close HPLC profile to that of fraction A34-1-9, the latter was selected for chromatography to obtain related compounds of A34-D2-2. A34-1-9 (306 mg) was further purified using two normal phase (Diol, 15 g) columns, and eluted with gradient solvent systems consisting of CH_2Cl_2 – EtOAc – MeOH and hexane - CH_2Cl_2 – EtOAc respectively. The chromatography was guided by both HPLC and ^{1}H NMR spectroscopy to locate related compounds. The fractions of interest were purified finally by semi-preparative HPLC (C_{18} column, reverse phase). Unfortunately, no pure related compounds were obtained. The reasons for failing to achieve pure related compounds were ascribed to the poor solubility of the major compound A34-D2-2 and the small content of related compounds: A34-D2-2 always appeared as a major compound in the fractions containing related compounds and the poor solubility of this compound made it very different to separate it from other components during chromatography.

3.3 Structural Elucidation of A34-D2-2

A34-D2-2 was obtained as pale-yellow powder. High-resolution electrospray mass spectrometry (HRESMS) showed the $[M+H^+]$ ion at 711.1181, indicating a molecular formula $C_{31}H_{31}N_6O_6S_4$ (calculated mass 711.1188). The ^{1}H NMR spectrum (Table 3.1) revealed signals for three methyl groups, four AB quartets, two singlets at δ_H 5.29 and 6.20 (each representing a proton), and nine aromatic protons. The heteronuclear single quantum coherence (HSQC) spectrum (Table 3.1) revealed four methylene groups. Two were assumed to be hydroxymethyl groups by chemical shifts (δ_C 61.2 and 60.6, δ_H 4.25 – 4.34). ^{13}C NMR spectrum by attached proton test (APT) indicated the presence of the following signals: three *N*-methyl groups (δ_C 27.4, C-17'; δ_C 27.5, C-13 and δ_C 28.2, C-16'), four methylene groups (δ_C 27.0, C-7'; δ_C 42.6, C-11; δ_C 60.6, C-12 and δ_C 61.2, C-15'), one aliphatic methine (δ_C 80.1, C-5), four amide carbonyls (δ_C 163.2, C-4; δ_C 165.5, C-1 and C-1' and δ_C 166.8, C-4'), five aliphatic quaternary carbons (δ_C 73.5, C-11a; δ_C 73.7, C-10b; δ_C 74.8, C-3'; δ_C 76.1,

C-3 and δ_C 76.6, C-6') and fourteen aromatic carbons (Table 3.1).

The appearance of an indole and an indoline fragment was suggested by the ^{1}H and ^{13}C NMR data. Analysis of ^{1}H-^{1}H correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY) (Table 3.1) and HSQC spectra led to the assignment of C-7 to C-10 and C-11' to C-14' (bold line in Figure 3.6). Analysis of the heteronuclear multiple bond coherence (HMBC) spectrum (Table 3.1) confirmed all the other assignments in the fragments. The principal correlations were as follows: H-7 (δ_H 6.77) to δ_C 126.5 (C-10a), H-8 (δ_H 7.27) to δ_C 148.3 (C-6a), H-5 (δ_H 6.20) to δ_C 73.7 (C-10b), δ_C 126.5 (C-10a) and δ_C 148.3 (C-6a), H-12' (δ_H 7.22) to δ_C 134.0 (C-10a'), H-11' (δ_H 7.30) and H-13' (δ_H 7.20) to δ_C 130.4 (C-14a'), H-9' (δ_H 7.19) to δ_C 134.0 (C-10a') and δ_C 107.6 (C-8') and H-14' (δ_H 7.65) to δ_C 107.6 (C-8') (Figure 3.6).

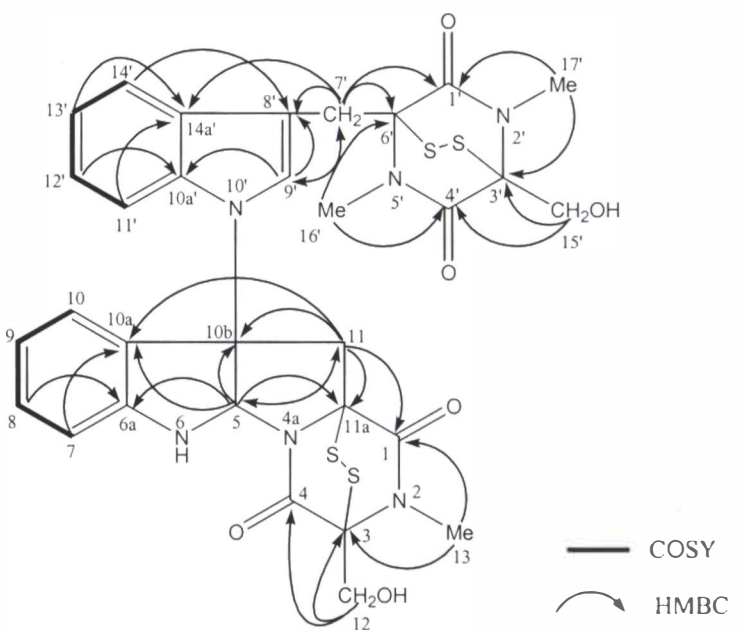


Figure 3.6 Structure for A34-D2-2

The connection of the functional groups was also demonstrated on the basis of HMBC correlations (Table 3.1). Two hydroxymethyl groups were connected to two amide carbonyl groups through a quaternary carbon respectively based on the long-range correlations of H-12 (δ_H 4.26 & 4.34) to δ_C 76.1 (C-3) and δ_C 163.2 (C-4) and H-15' (δ_H 4.25 & 4.33) to δ_C 74.8 (C-3') and δ_C 166.8 (C-4') (Figure 3.6). The long-range correlations of *N*-methyl protons at δ_H 3.17 (H-13) to carbons at δ_C 76.1 (C-3) and δ_C

165.5 (C-1) and the other *N*-methyl protons at δ_H 3.15 (H-17') to carbons at δ_C 74.8 (C-3') and δ_C 165.5 (C-1') led to the connection of C-1 to N-2 then to C-3 and C-1' to N-2' then to C-3' respectively. The third *N*-methyl group was assigned by the long-range correlations of H-16' (δ_H 2.95) to δ_C 76.6 (C-6') and δ_C 166.8 (C-4'). The long-range correlations of H-7' (δ_H 3.69 & 3.85) to δ_C 107.6 (C-8'), δ_C 76.6 (C-6') and δ_C 165.5 (C-1') allow the connection of C-8' to C-1' through C-7' and C-6'. The connection of C-5 to C-11a through a nitrogen was suggested by the chemical shifts of the methine group (δ_C 80.1, δ_H 6.20) and the long-rang correlation between H-5 (δ_H 6.20) to δ_C 42.6 (C-11) and δ_C 73.5 (C-11a). The connection of C-11 to the indoline group and to C-1 through C-11a was assumed by the long-range correlation of H-11 (δ_H 3.09 & 4.40) to δ_C 73.7 (C-10b), δ_C 126.5 (C-10a), δ_C 80.1 (C-5), δ_C 73.5 (C-11a) and δ_C 165.5 (C-1). The assignment of four sulfur atoms was based mainly on the chemical shifts of C-3, C-11a, C-3' and C-6' and the molecular formula. Thus, a planar structure as shown in Figure 3.6 was proposed for compound A34-D2-2.

On a literature search, compound A34-D2-2 was identical to chetomin, an epipolythiodioxopiperazine previously isolated from several species of *Chaetomium*.[102] The stereostructure was decided as shown in Figure 3.7 by comparing the chemical shifts and coupling constants with those in the literature.[86,88,103]

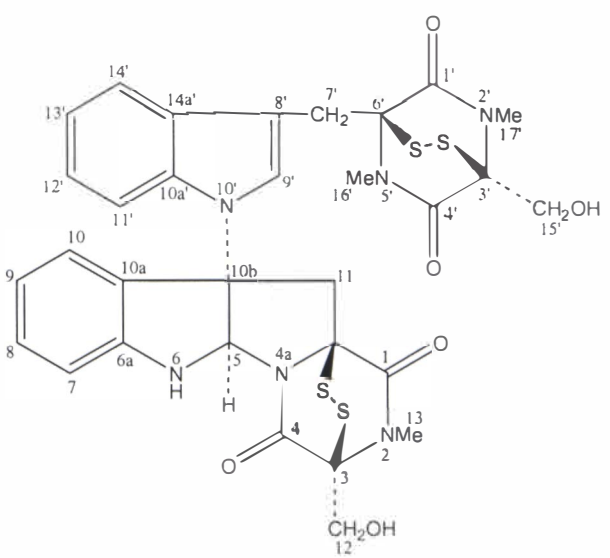


Figure 3.7 Structure of chetomin

Table 3.1 ^{1}H, ^{13}C, HSQC, COSY, TOCSY and HMBC NMR data for A34-D2-2[a]

no.	^{13}C δ	^{1}H δ[b]	COSY	TOCSY	HMBC
1	165.5 (C)[c]				
3	76.1 (C)				
4	163.2 (C)				
5	80.1 (CH)	6.20 (1H, s)			42.6, 73.5, 73.7, 111.1, 126.5, 148.3
6		5.29 (1H, s) (N-H)			
6a	148.3 (C)				
7	111.1 (CH)	6.77 (1H, d, 8.0)	7.27	6.93, 7.27, 7.32	120.3, 126.5
8	131.4 (CH)	7.27 (1H, t. 7.8)	6.77, 6.93	6.77, 6.93	111.1, 120.3, 125.0, 148.3
9	120.3 (CH)	6.93 (1H, t, 7.5)	7.27, 7.32	6.77, 7.27, 7.32	73.7, 111.1, 125.0, 126.5, 131.4, 148.3
10	125.0 (CH)	7.32 (1H, d, 7.6)	6.93	6.77, 6.93	73.7, 111.1, 120.3, 131.4, 148.3
10a	126.5 (C)				
10b	73.7 (C)				
11	42.6 (CH_2)	4.40 (1H, d, 16.0)	3.09	3.09	73.5, 73.7, 126.5, 165.5
		3.09 (1H, d, 15.0)	4.40	4.40	73.5, 73.7, 80.1, 126.5, 163.2
11a	73.5 (C)				
12	60.6 (CH_2)	4.26 (1H, d, 12.5)	4.34	4.34	76.1, 163.2
		4.34 (1H, d, 13.0)	4.26	4.26	76.1, 163.2
13	27.5 (CH_3)	3.17 (3H, s)			60.6, 76.1, 165.5
1'	165.5 (C)				
3'	74.8 (C)				
4'	166.8 (C)				
6'	76.6 (C)				
7'	27.0 (CH_2)	3.69 (1H, d, 15.5)	3.85, 7.19	3.85, 7.19	76.6, 107.6, 127.2, 130.4, 165.5
		3.85 (1H, d, 16.0)	3.69, 7.19	3.69, 7.19	76.6, 107.6, 127.2, 130.4, 165.5
8'	107.6 (C)				
9'	127.2 (CH)	7.19 (1H, s)	3.69, 3.85	3.69, 3.85	27.0, 73.7, 107.6, 134.0
10a'	134.0 (C)				
11'	111.4 (CH)	7.30 (1H, m)	7.22	7.20, 7.22, 7.65	120.6, 122.8, 130.4, 134.0
12'	122.8 (CH)	7.22 (1H, m)	7.30	7.30, 7.65	111.4, 119.1, 134.0
13'	120.6 (CH)	7.20 (1H, m)	7.65	7.30, 7.65	111.4, 119.1, 122.8, 130.4
14'	119.1 (CH)	7.65 (1H, d, 9.0)	7.20	7.20, 7.22, 7.30	107.6, 122.8, 130.4, 134.0
14a'	130.4 (C)				
15'	61.2 (CH_2)	4.25 (1H, d, 12.5)	4.33	4.33	74.8, 166.8
		4.33 (1H, d, 13.0)	4.25	4.25	74.8, 166.8
16'	28.2 (CH_3)	2.95 (3H, s)			27.0, 76.6, 165.5, 166.8
17'	27.4 (CH_3)	3.15 (3H, s)			61.2, 74.8, 165.5

[a] ^{1}H NMR spectra recorded at 500 MHz in $CDCl_3$, ^{13}C NMR spectra recorded at 75 MHz in $CDCl_3$.
[b] ^{1}H chemical shift values followed by number of protons, multiplicity and coupling constant (*J*/Hz).
[c] primary, secondary, tertiary and quaternary carbons, assigned by APT.

Biological Activity:

P388 activity: IC_{50} = 11.2 ng/mL

Antimicrobial activity: 4 mm inhibition zone against *Bacillus subtilis* at a concentration of 40 μg/disk.

Antiviral activity: CYT 4+, TYP 4/5 for *Herpes simplex virus*, CYT 4+, TYP 4/5 for *Polio virus* at a concentration of 30 μg/disk.

Chetomin is an antibiotic discovered in 1944.[104] It has also been associated with poor growth in young ruminants.[105] Chetomin has been reported to show high antibacterial, antifungal, antiviral and antitumour activity.[92,105,106] It is bacteriostatic for a series of bacteria and is mainly active against Gram-positive bacteria.[90] Chetomin is one of the most active compounds among epipolythiodioxopiperazines in the assay against the HeLa cells. It has potent cytotoxicity with IC_{50} values of 0.02 μg/mL.[88] The disulfide bond in chetomin is thought to be essential for its activity.

3.4 Discussion

In the literature,[103] the assignment of ^{13}C and ^{1}H signals was made using known chemical shift trends and published chemical shift data for indoline, chaetocin, and substituted indoles. Chemical shifts and spin-spin couplings for the benzene-type protons were obtained by spectral simulation techniques. Because 2D-NMR spectra (COSY, TOCSY, HSQC and HMBC) were acquired in this experiment, reassignments for H-9', C-3' and C-6'were made and the ambiguities in the NMR assignment of C-8 (H-8) and C-10 (H-10), C-12 and C-15', C-13 (H-13), C-16' (H-16') and C-17' (H-17') in the literature were removed.

Part II *Chaetomium* sp. (N60)

3.5 Taxonomy of *Chaetomium* sp. (N60)

Strain No. N60 was isolated from saline sand in New Zealand.

Morphological and cultural characteristics: Colonies on malt extract agar (MEA) 58-67 mm in diameter (7 days, 25 °C, Figure 3.8), with a buff aerial mycelium, reverse pale. Colonies on 25% glycerol nitrate agar (G25N) 2-3 mm diameter (7 days, 25 °C). No growth at 5 °C. At 37 °C, colonies 15-16 mm in diameter (MEA, 7 days).

Ascomata maturing at 7-10 days, brown, superficial, spherical or ovate, ostiolate, 145-238 x 173-252 µm (Figure 3.8); ascomatal hairs numerous, unbranched, undulate or coiled, septate, pale greenish brown; asci clavate, 22-33 x 11-17 µm, 8 spored, evanescent; ascospores limoniform, brownish when mature, containing numerous droplets, 6-8 x 7-9 µm, with an apical germ pore (Figure 3.8).

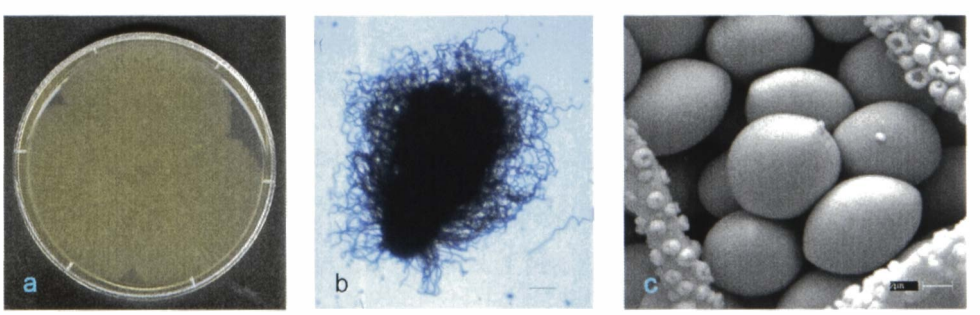


Figure 3.8 *Chaetomium globosum* (a) colonies on MEA (7 days, 25 °C); (b) ascocarp (stained with lactophenol cotton blue); (c) ascospores. Bars: b = 100 µm, c = 2 µm

After comparing the characteristics of this strain with descriptions by Von Arx *et al.*[76] and Pitt and Hocking,[107] strain No. N60 was identified as *Chaetomium globosum* Kunze.

3.6 Culturing and Extraction of *Chaetomium globosum*

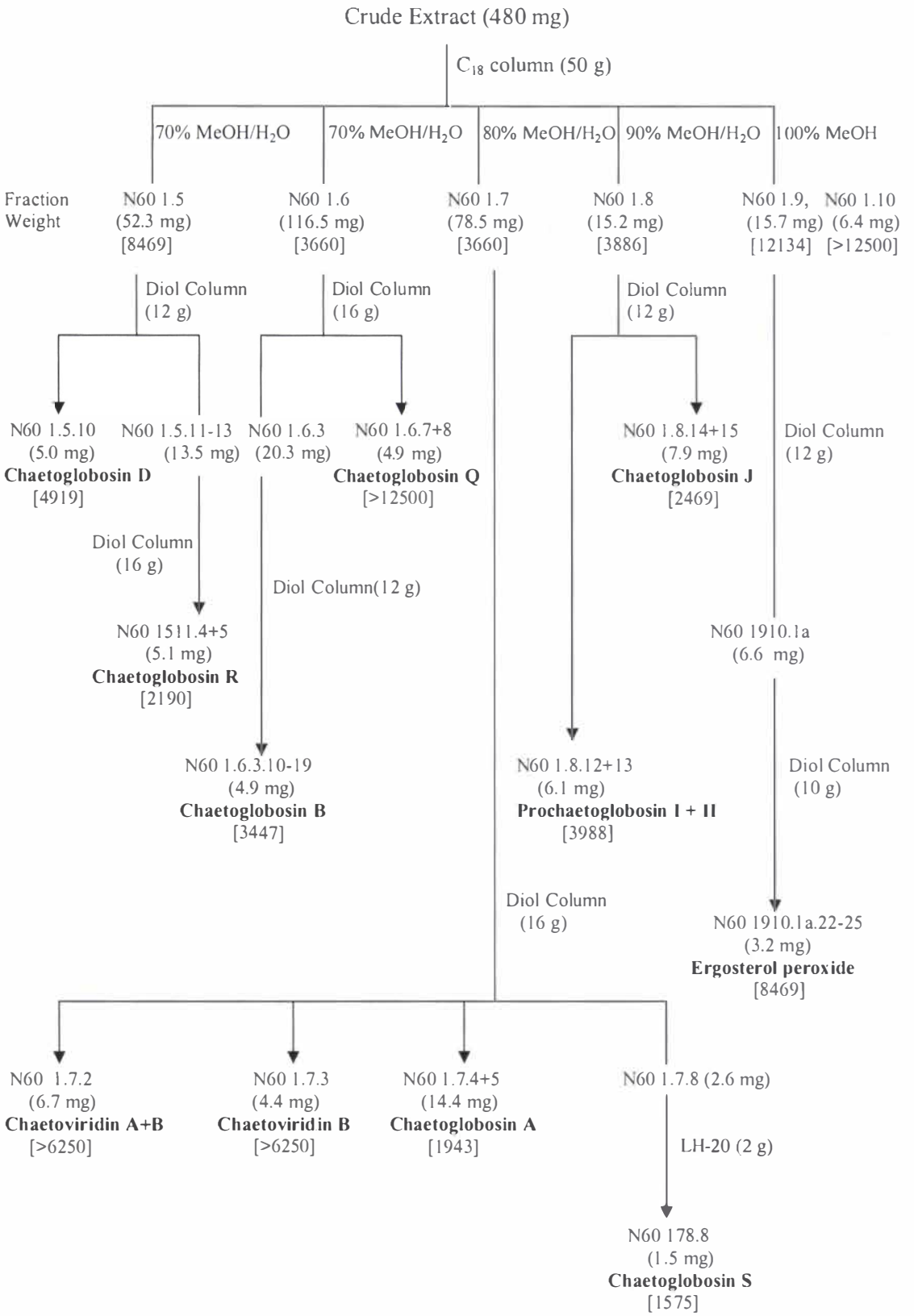
C. globosum (N60) was inoculated onto potato dextrose agar (PDA) and incubated for five days at 25 °C in darkness. Mycelial disks (8 mm in diameter) were cut from the growing margin of the colony and transferred into 2 litres of half strength malt extract broth. The broth was incubated for four weeks at 26 °C under agitating conditions. The broth was then filtered through Celite 545 under suction and the mycelium and filtrate extracted four times with EtOAc respectively. The extracts were dried over $MgSO_4$ and evaporated under reduced pressure to give a mixture of crude metabolites (mycelial extract: yellowish brown, 234 mg; filtrate extract: brownish red, 294 mg). On the assay against the P388 murine leukaemia cell line, the filtrate and mycelial extracts showed similar activity (IC_{50} 1942 and 2325 ng/mL, respectively). Because the HPLC profiles of mycelium extract and filtrate extract were identical, they were combined before further chromatography.

3.7 Chromatography of *Chaetomium globosum*

A general outline of the chromatography is given in **Scheme 3.2**.

The crude extract (480 mg) was separated on a C_{18} column (reverse-phase), eluted with a stepped gradient from 10% MeOH/H_2O to MeOH and then CH_2Cl_2. Fourteen fractions were collected. Fractions N60 1.5, 1.6, 1.7 and 1.8 (eluted with 70% MeOH/H_2O, 70% MeOH/H_2O, 80% MeOH/H_2O and 90% MeOH/H_2O respectively) showed strong activities against the P388 cell line (IC_{50} 3660 – 8469 ng/mL). Fraction N60 1.9 (eluted with 100% MeOH) exhibited weak P388 activity (IC_{50} 12134 ng/mL). The UV profiles of major compounds in fractions N60 1.5, 1.6, 1.7 and 1.8 were similar, indicating a series of closely related compounds responsible for the observed activity. Therefore, further purification was guided mainly by UV profiles in the HPLC and ^1H NMR spectra.

Scheme 3.2



[ ]: P388 activity (IC_{50} = ng/mL)

3.7.1 Separation of fraction N60 1.6

Fraction N60 1.6 (116.5 mg, Scheme 3.2) was further chromatographed on 16 g of Diol (normal phase), using a gradient system consisting of CH_2Cl_2-EtOAc-MeOH. Fifteen subfractions were collected. A pure compound, N60 1.6.7+8 (4.9 mg), was achieved from subfractions N60 1.6.7 and 1.6.8, eluted with 50% and 80% EtOAc/CH_2Cl_2 respectively. Subfractions N60 1.6.3 and N60 1.6.4, eluted with 5% EtOAc/CH_2Cl_2, contained a major compound of crude N60 1.6. Because subfraction N60 1.6.3 was purer than N60 1.6.4, the former was used to obtain the major compound. Subfraction N60 1.6.3 was applied on another Diol column (12 g) and eluted with 70% CH_2Cl_2/hexanes isocratically. Thirty-four fractions were collected. Pure compound N60 1.6.3.10-19 (4.9 mg) was obtained in the fractions N60 1.6.3.10 to N60 1.6.3.19.

The structural elucidation of N60 1.6.7+8 and N60 1.6.3.10-19 is discussed in sections **3.8.3** and **3.8.1** .

3.7.2 Separation of fraction N60 1.7

Fraction N60 1.7 (78.5 mg) was separated with a normal phase (Diol, 16 g) column, using a stepped gradient solvent system from CH_2Cl_2 to EtOAc then to MeOH. Seventeen subfractions were collected. Subfraction N60 1.7.3, eluted with 100% CH_2Cl_2, afforded a pure compound (N60 1.7.3, 4.4 mg). Subfractions N60 1.7.4 and 1.7.5, eluted with 5% EtOAc/CH_2Cl_2, gave another pure compound (N60 1.7.4+5, 14.4 mg). N60 1.7.4+5 was the major compound of crude N60 1.7.

^{1}H NMR spectra indicated that subfraction N60 1.7.8, eluted with 20% EtOAc/CH_2Cl_2, contained a compound closely related to N60 1.7.4+5. Subfraction N60 1.7.8 (2.6 mg) was further chromatographed by a LH-20 (2 g) column using 100% MeOH as the eluent. The expected pure compound (N60 178.8, 1.5 mg) was achieved in fraction N60 178.8.

The HPLC profile and ^{1}H NMR spectrum indicated that subfraction N60 1.7.2 was a mixture of nearly equal amounts of compound N60 1.7.3 and a related compound, namely N60 1.7.2A. Because no P388 activity was detected in this fraction and ^{1}H

NMR signals of compound N60 1.7.2A and N60 1.7.3 did not overlap, identification of compound N60 1.7.2A was made by removal of the signals of N60 1.7.3 from the ^{1}H NMR spectrum of N60 1.7.2. Therefore, no further purification was necessary for subfraction N60 1.7.2.

The structural elucidation of compounds N60 1.7.4+5, N60 178.8, N60 1.7.3 and N60 1.7.2A is discussed in sections **3.8.2** , **3.8.5** , **3.8.6** and **3.8.7** respectively.

3.7.3 Separation of fraction N60 1.8

The fraction N60 1.8 (15.2 mg) was chromatographed on 12 g of Diol, using a gradient solvent system from hexanes to EtOAc, then to MeOH. Twenty-nine subfractions were collected and combination of the subfractions was based on analysis of ^{1}H NMR spectra. Pure compound N60 1.8.14+15 (7.9 mg) was afforded in the subfractions eluted with 100% EtOAc. Subfractions N60 1.8.12 and N60 1.8.13, eluted with 80% EtOAc/hexanes, were combined (N60 1.8.12+13). The HPLC profile and ^{1}H NMR spectrum indicated that subfraction N60 1.8.12+13 was relatively pure, and contained two compounds (namely N60 1.8.12+13 A and B), structurally related to compound N60 1.8.14+15. The ratio of the two compounds was nearly 1:1. Because ^{1}H NMR signals of the two compounds could be distinguished from each other in the spectrum, it was concluded that the structural elucidation could be done by combining MS and a series of 2D NMR spectra. Due to the small mass (6.1 mg) of the fraction and the fact that they were known compounds (see section **3.8.2**), no further purification was carried out for this subfraction.

The structural elucidation of the three compounds is discussed in section **3.8.2** .

3.7.4 Separation of fraction N60 1.5

Fraction N60 1.5 (52.3 mg) was applied onto a Diol column (12 g) and eluted with a gradient solvent system from 10% EtOAc/hexanes to 100% EtOAc then to MeOH. Thirty subfractions were collected and combination of the subfractions was based on analysis of ^{1}H NMR spectra. A pure compound (N60 1.5.10, 5.0 mg) was isolated in the subfraction eluted with 80% EtOAc/hexanes. Subfractions N60 1.5.11, 12 and 13, eluted with 90% EtOAc/hexanes, contained the same compound structurally related to

compound N60 1.5.10. The three subfractions were combined (13.5 mg) and further purified on another Diol column (16 g). The column was eluted with a gradient system from 10% EtOAc/hexanes to 100% EtOAc and nine fractions were collected. A pure compound (N60 1511. 4+5, 5.1 mg) was achieved in the fractions N60 1511.4 and N60 1511.5, eluted with 70% EtOAc/hexanes.

The structural elucidation of N60 1.5.10 and N60 1511.4+5 is discussed in sections **3.8.2** and **3.8.4** .

3.7.5 Separation of fractions N60 1.9 and N60 1.10

Fraction N60 1.9 showed mild activity against the P388 cell line (IC_{50} 12134 ng/mL) while fraction N60 1.10 was inactive in the assay (IC_{50} >12500 ng/mL). Because the ^1H NMR spectra of these two fractions were identical, they were combined for further purification.

The combined fractions (22.1 mg) were chromatographed on a 12 g Diol (normal phase) column, using a gradient solvent system from hexanes to EtOAc, then to MeOH as the eluent. A total of twenty-eight subfractions was collected and combined after analysis by ^1H NMR spectroscopy. The compound of interest was collected in subfraction N60 1910.1a (eluted with 100% hexanes to 30% EtOAc/hexanes). This subfraction (6.6 mg) was further purified on Diol (10 g), eluted with a gradient from 100% hexanes to 50% EtOAc/hexanes. Twenty-six fractions were collected. Pure compound N60 1910.1a.22-25 (3.2 mg) was obtained in the fractions eluted with 30% EtOAc/hexanes.

The structural elucidation of N60 1910.1a.22-25 is discussed in section **3.8.8** .

3.8 Structural Elucidation

3.8.1 Structural elucidation of compound N60 1.6.3.10-19

N60 1.6.3.10-19 was one of the main compounds isolated from the culture of *Chaetomium globosum* (N60).

The molecular formula of N60 1.6.3.10-19 was determined to be $C_{32}H_{36}O_5N_2$ based

on analysis of the HRESMS (found: 529.2714 [M+H$^+$], calcd: 529.2702) and the ^{13}C NMR spectrum (Table 3.2).

The ^{1}H NMR and HSQC spectra (Table 3.2) disclosed the presence of the following groups: a secondary methyl (δ_H 1.02, H-16'), three allylic methyls (δ_H 1.37, H-18'; δ_H 1.65, H-11 and δ_H 1.73, H-12), two trans -CH=CH- groups (δ_H 6.75 and 7.75, J =16 Hz, H-21 and H-22; δ_H 6.19 and 5.36, J =15 Hz, H-13 and H-14), two methylenes (δ_H 2.65 and 2.88, H-10; δ_H 2.06 and 2.32, H-15), six methines (δ_H 3.55, H-3; δ_H 3.42, H-4; δ_H 3.94, H-7; δ_H 2.04, H-8; δ_H 2.52, H-16 and δ_H 5.07, H-19) including two oxymethines (δ_C 68.9, C-7 and δ_C 82.2, C-19) and an olefinic proton (δ_H 5.61, H-17). The presence of a 3-substituted indole group was also deduced by comparing the ^{13}C NMR data (see Table 3.2).

The identity of the remaining groups was revealed by the analysis of APT: two conjugated ketones (δ_C 197.3, C-23 and δ_C 201.3, C-20), an amide (δ_C 173.2, C-1), an aliphatic quaternary carbon (δ_C 61.5, C-9) and three olefinic carbons (δ_C 126.3, C-5; δ_C 132.0, C-6 and δ_C 132.5, C-18).

Close inspection of the ^{1}H spectra of N60 1.6.3.10-19 by COSY experiment (Table 3.2) led to the partial structural units as shown by bold-faced lines in Figure 3.9. The assignments of C-5, C-6, C-11 (δ_C 18.1), C-12 (δ_C 14.1) and C-18 were made by long-range correlations (CIGAR) between H-3 to C-5, H-4 to C-6, H-12 to C-7 and H-18' to C-18 (Figure 3.9).

The assignments of C-1'a (δ_C 136.5), C-3' (δ_C 111.2) and C-3'a (δ_C 127.0) in the indole group were determined by long-range correlations in the CIGAR spectrum (Table 3.2), mainly between H-2' (δ_H 6.99) and H-6' (δ_H 7.21) to C-1'a, H-4' (δ_H 7.48) to C-3'and H-5' (δ_H 7.13) to C-3'a (Figure 3.9).

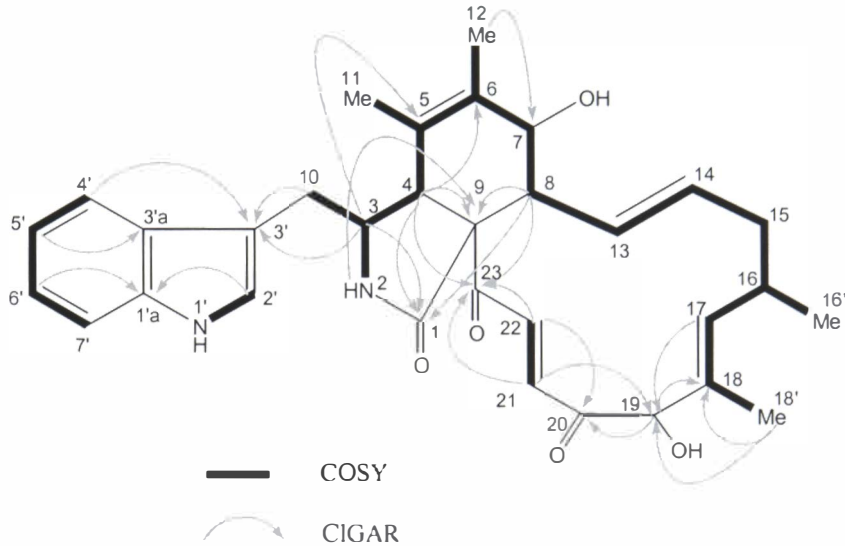


Figure 3.9 Planar structure of compound N60 1.6.3.10-19

The long-range correlations of H-4 and H-8 to C-9 led to the connection of a six-membered ring from C-4 to C-9. The long-range correlations of H-3, H-4 and H-8 to C-1 and H-2 (δ_H 5.68) to C-9 allowed the connection of N-2 to C-1 then to C-9. The connection of C-18 to C-21 through C-19 and C-20 was based mainly on the long-range correlations of H-19 to C-18, H-17, H-18' and H-21 to C-19 and H-19 and H-22 to C-20. The connection of C-23 to C-9 and C-22 was indicated by the long-range correlations of H-4, H-8, H-21 and H-22 to C-23. Finally, the connection of C-10 to the indole group was made by the long-range correlations of H-10a, H-10b and H-3 to C-3'.

On the basis of these findings, the planar structure in Figure 3.9 was proposed for compound N60 1.6.3.10-19. From a literature search, this compound was identified as chaetoglobosin B, previously isolated from *Chaetomium* spp.[81] The stereochemistry of N60 1.6.3.10-19 was determined as shown in Figure 3.10 by comparison with the literature.[81,108,109] Because a high resolution NMR instrument was used and 2D-NMR data (COSY, HSQC and CIGAR) were obtained for compound N60 1.6.3.10-19, the ambiguities in the NMR assignment in the literature were removed by our own more precise data.

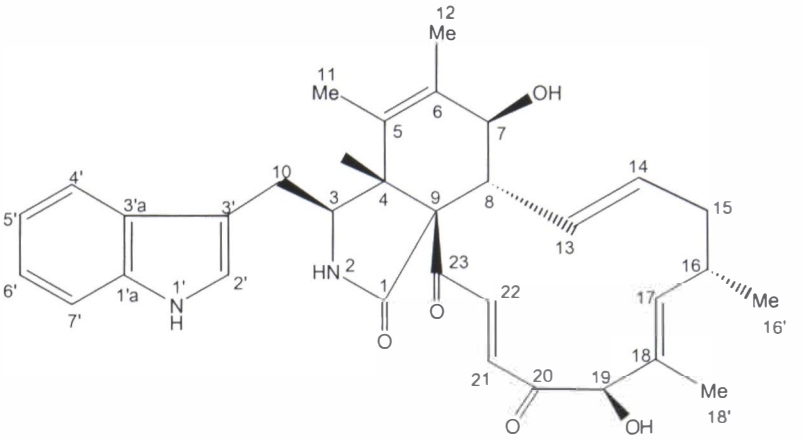


Figure 3.10 Structure of chaetoglobosin B

Table 3.2 ^{1}H, ^{13}C, HSQC, COSY and CIGAR NMR data for N60 1.6.3.10-19[a]

no.	^{13}C δ	^{1}H δ[b]	COSY	CIGAR[d]
1'		8.09 (1H, br s) (N-H)	H-2'	C-2'(w), C-3', C-3'a
1'a	136.5 (C)[c]			
2'	123.2 (CH)	6.99 (1H, s)	H-1'	C-1'a, C-3', C-3'a
3'	111.2 (C)			
3'a	127.0 (C)			
4'	118.6 (CH)	7.48 (1H, d, 7.5)	H-5'	C-1'a, C-3', C-3'a (w), C-6'
5'	120.1 (CH)	7.13 (1H, t, 7.5)	H-4', H-6'	C-3'a, C-7'
6'	122.7 (CH)	7.21 (1H, t, 8.0)	H-5', H-7'	C-1'a, C-4', C-7'
7'	111.7 (CH)	7.37 (1H, d, 8.5)	H-6'	C-3'a, C-5', C-6'
1	173.2 (C)			
2		5.68 (1H, br s) (N-H)	H-3	C-3, C-4, C-9
3	58.4 (CH)	3.55 (1H, m)	H-2, H-4, H-10a, H-10b	C-1, C-4, C-5, C-3'(w)
4	47.9 (CH)	3.42 (1H, br s)	H-3, H-11, H-12	C-1, C-3, C-5, C-6, C-9, C-10, C-11, C-23
5	126.3 (C)			
6	132.0 (C)			
7	68.9 (CH)	3.94 (1H, br d, 10)	H-4 (w), H-7' (7-OH), H-8, H-11, H-12	
7'		1.86 (1H, br s) (O-H)	H-7	
8	52.7 (CH)	2.04 (1H, m)	H-7, H-13	C-1, C-6 (w), C-7, C-9, C-13, C-14, C-23
9	61.5 (C)			
10	33.3 (CH_2)	H10a: 2.88 (1H, dd, 14, 5)	H-3, H-10b	C-2', C-3', C-3'a, C-3, C-4
		H10b: 2.65(1H, dd, 14, 9)	H-3, H-10a	C-2', C-3', C-3'a, C-3, C-4
11	18.1 (CH_3)	1.65 (3H, br s)	H-4, H-7	C-4, C-5, C-6
12	14.1 (CH_3)	1.73 (3H, br s)	H-4, H-7	C-4, C-5, C-6, C-7
13	127.9 (CH)	6.19 (1H, dd, 15, 10)	H-8, H-14	C-8, C-15(w),
14	137.4 (CH)	5.36 (1H, m)	H-13, H-15a, H-15b	C-8 (w)
15	41.6 (CH_2)	H15a: 2.06 (1H, m)	H-14, H-15b, H-16	C-13, C-14, C-16(w)
		H15b: 2.32 (1H, br d, 14)	H-14(w), H-15a, H-16(w)	
16	32.5 (CH)	2.52 (1H, br m)	H-15a, H-15b, H-16', H-17	
16'	21.3 (CH_3)	1.02 (3H, d, 6.5)	H-16	C-15, C-16, C-17
17	140.3 (CH)	5.61 (1H, d, 10)	H-16, H-18'	C-15, C-16(w), C-18', C-19
18	132.5 (C)			
18'	10.9 (CH_3)	1.37 (3H, s)	H-17	C-17, C-18, C-19
19	82.2 (CH)	5.07 (1H, d, 5)	H-19' (19-OH)	C-17, C-18, C-18', C-20
19'		3.82 (1H, d, 4.5) (O-H)	H-19	
20	201.3 (C)			
21	133.3 (CH)	6.75 (1H, d, 16)	H-22	C-19(w), C-22, C-23
22	136.2 (CH)	7.75 (1H, d, 16)	H-21	C-20, C-23
23	197.3 (C)			

[a] ^{1}H NMR spectra recorded at 500 MHz in $CDCl_3$, ^{13}C NMR spectra recorded at 125 MHz in $CDCl_3$. [b] ^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by number of protons, multiplicity and coupling constant (J/Hz). [c] primary, secondary, tertiary and quaternary carbons, assigned by APT. [d] long range ^{1}H-^{13}C correlation observed in the CIGAR experiment.

3.8.2 Structural elucidation of five related compounds

3.8.2.1 Structural elucidation of compound N60 1.7.4+5

Compound N60 1.7.4+5 was another major compound isolated from the culture of *Chaetomium globosum* (N60).

The HRESMS indicated that compound N60 1.7.4+5 had the same molecular formula ($C_{32}H_{36}O_5N_2$) as that of chaetoglobosin B. The general features of its ^{1}H and ^{13}C NMR spectra (Table 3.3, Table 3.4) closely resembled those of chaetoglobosin B (Table 3.2) except for the differences in the region of the six-membered ring. Two allyllic methyl groups (δ_H 1.65, 1.73) in chaetoglobosin B were replaced by a secondary methyl group (δ_H 1.24, H-11) at C-5 and a tertiary methyl group (δ_H 1.29, H-12) at C-6 in N60 1.7.4+5. The C-5, C-6 olefinic carbon signals and a secondary alcohol group at C-7 in chaetoglobosin B were absent, and instead a methine group (δ_C 36.3, δ_H 1.84) and an aliphatic quaternary carbon (δ_C 58.3) appeared in N60 1.7.4+5. The presence of a trisubstituted expoxide (C-6 and C-7) was suggested and the structure depicted in Figure 3.11 was proposed. This structure was further confirmed by a series of 2D NMR experiments (COSY, HSQC and CIGAR). A literature search revealed this compound to be chaetoglobosin A, previously isolated from *Chaetomium* spp.[81] The stereochemistry of N60 1.7.4+5 was determined by comparison with the literature.[81,108,109]

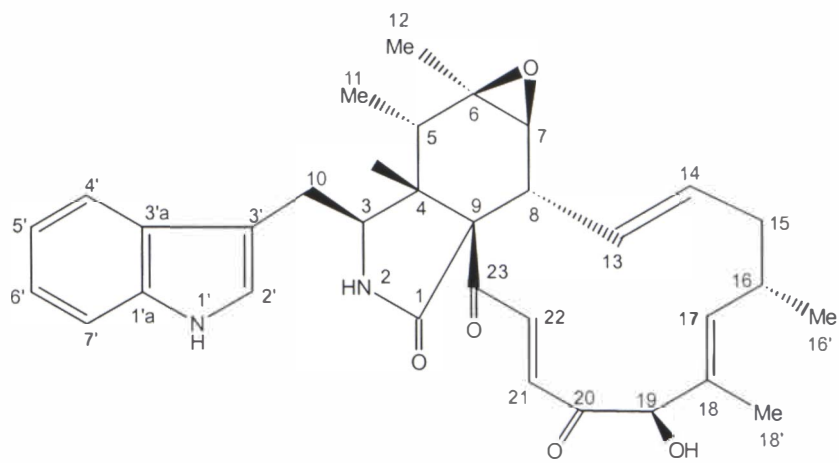


Figure 3.11 Structure of chaetoglobosin A (N60 1.7.4+5)

3.8.2.2 Structural elucidation of compound N60 1.5.10

Compound N60 1.5.10 had the same molecular formula ($C_{32}H_{36}O_5N_2$) as chaetoglobosin B, as deduced from HRESMS (found: 529.2702 [M+H$^+$], calcd: 529.2702). The ^{1}H and ^{13}C NMR spectra (Table 3.3, Table 3.4) showed close correspondence with those of chaetoglobosin B except for the absence of an allylic methyl group at C-6 and the presence of a methine (δ_C 32.7, δ_H 2.84) and a terminal methylene group (δ_C 113.4, δ_H 5.21 & 5.44). The methine group (C-5, H-5) was assigned by the COSY coupling of H-5 (δ_H 2.84) to H-4 and H-11. The long-range correlations (CIGAR) of the terminal methylene protons to C-5 and C-7 led to the connection of C-6 (δ_C 148.7) to C-12 (δ_C 113.4). The chemical structure of N60 1.5.10 was determined as shown in Figure 3.12. A literature search revealed this compound to be chaetoglobosin D with the stereochemistry assigned after comparison to the literature.[79,80,109]

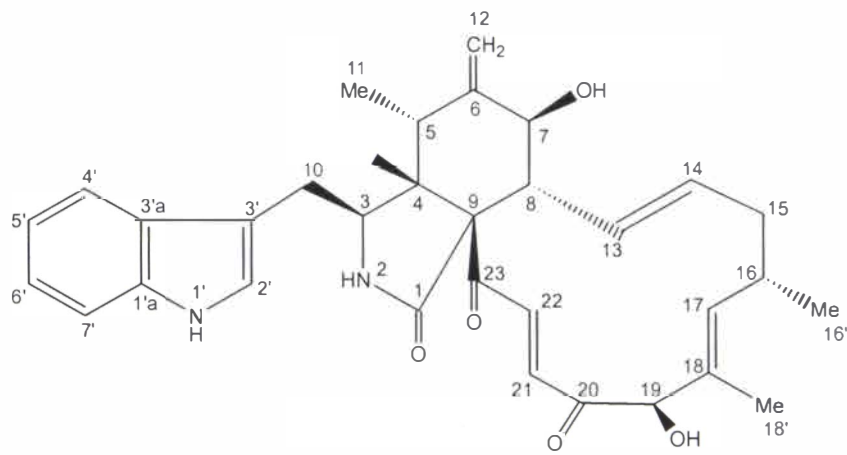


Figure 3.12 Structure of chaetoglobosin D (N60 1.5.10)

3.8.2.3 Structural elucidation of compound N60 1.8.14+15

The molecular formula of compound N60 1.8.14+15 was determined to be $C_{32}H_{36}O_4N_2$ based on HRESMS (found: 513.2749 [M+H$^+$], calcd: 513.2753). This formula corresponded to a deoxygenated congener of chaetoglobosin B. Comparison of the ^{1}H and ^{13}C NMR signals (Table 3.3, Table 3.4) revealed the presence of a secondary methyl group (δ_H 1.43) at C-5, the appearance of an olefinic proton (δ_H 5.30) at C-7, and the absence of the C-7 alcohol of chaetoglobosin B. The assignment

of C-5 (H-5) was based on COSY correlations between H-5 (δ_H 2.47) to H-4 and H-11. The assignments of C-6 and C-7 (H-7) were made by the long-range couplings (CIGAR) of H-4 to C-6 (δ_C 141.2) and H-12 to C-7 (δ_C 125.8). The latter was also supported by the COSY correlation between H-7 (δ_H 5.30) to H-8 and H-12. Based on the information from COSY, HSQC and CIGAR spectra, the structure of compound N60 1.8.14+15 was suggested as depicted in Figure 3.13. This compound was found to be identical with chaetoglobosin J, previously isolated from *Chaetomium* spp.,[80] and the stereochemistry of N60 1.8.14+15 determined by comparison of the NMR data with the literature.[80,109]

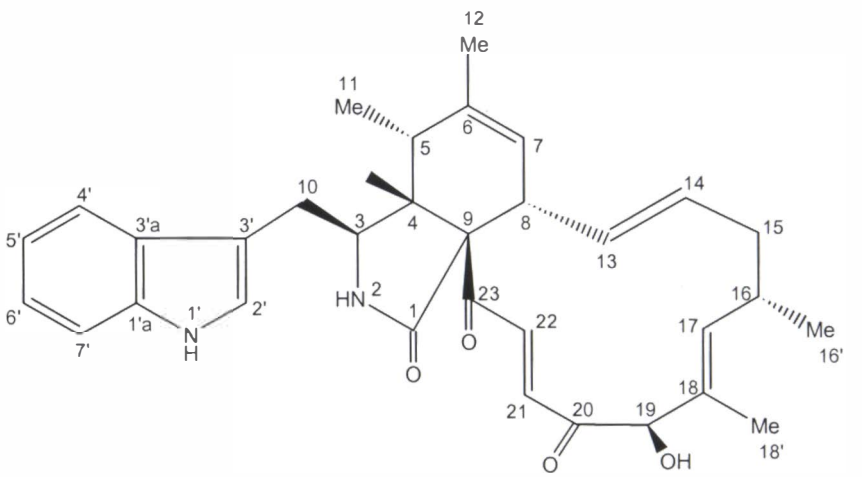


Figure 3.13 Structure of chaetoglobosin J (N60 1.8.14+15)

3.8.2.4 Structural elucidation of compound N60 1.8.12+13A

The molecular formula of compound N60 1.8.12+13A was determined to be $C_{32}H_{38}O_2N_2$ by HRESMS (found: 483.2995 [M+H$^+$], calcd: 483.3012). Its close relationship to chaetoglobosin J (N60 1.8.14+15) was revealed by the ^{1}H and ^{13}C NMR spectra (Table 3.3, Table 3.4) in which the majority of the chemical shifts were practically identical. Since the difference between chaetoglobosin J and N60 1.8.12+13A was in the number of hydrogen and oxygen atoms, N60 1.8.12+13A was assumed to be the reduced form of chaetoglobosin J. After comparing the ^{13}C and ^{1}H NMR spectra of chaetoglobosin J and N60 1.8.12+13A (Table 3.3, Table 3.4), it was found that the difference between the structures were concentrated around C-19 (H-19) and C-20 (H-20). The oxygenated carbon at C-19 (δ_C 81.5) and the carbonyl

group at C-20 (δ_C 201.2) in chaetoglobosin J were changed to δ_C 35.8 and δ_C 28.7 respectively. These two carbons were determined to be two methylene carbons by HSQC correlations (δ_H 2.36 and δ_H 2.13 to δ_C 35.8, δ_H 2.38 and δ_H 2.39 to δ_C 28.7), and the double triplet (H-21) at δ_H 6.79 coupling with a methylene group at δ_H 2.38, 2.39 supported this elucidation. On the basis of the above observation, the structure of compound N60 1.8.12+13A was proposed as shown in Figure 3.14. A literature search found this compound to be prochaetoglobosin I, previously isolated from a cell free system *of Chaetomium subaffine*, treated with cytochrome P-450 inhibitors. The stereochemistry was decided by comparison with the data in the literature.[110,111]

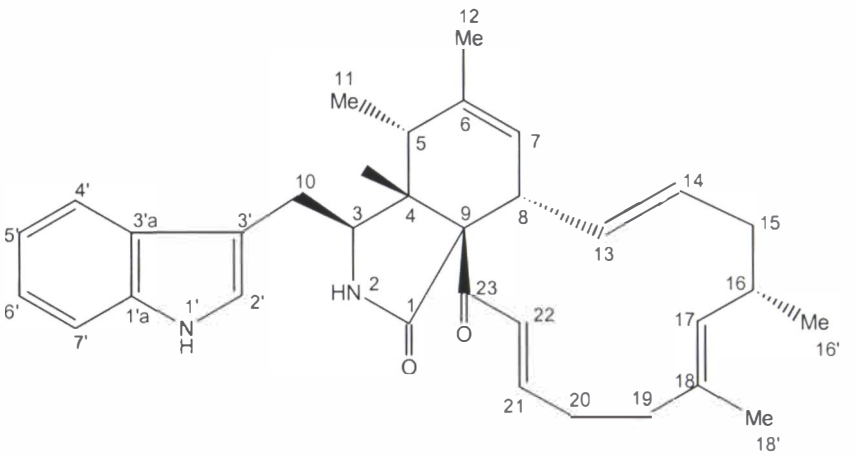


Figure 3.14 Structure of prochaetoglobosin I (N60 1.8.12+13A)

3.8.2.5 Structural elucidation of compound N60 1.8.12+13B

The molecular formula of compound N60 1.8.12+13B was deduced as $C_{32}H_{36}O_3N_2$ by HRESMS (found: 497.2796 [M+H$^+$], calcd: 497.2804), a deoxygenated congener of chaetoglobosin J (N60 1.8.14+15). Comparison of the ^{1}H and ^{13}C NMR spectra of N60 1.8.12+13B with those of chaetoglobosin J (Table 3.3, Table 3.4) noted that the differences between the structures lay at C-19. The HSQC spectra revealed the oxygenated methine group at C-19 (δ_C 81.5, δ_H 5.10) in chaetoglobosin J was replaced by a methylene group (δ_C 53.2, δ_H 3.05 and 3.70). This was also supported by the isolated germinally signals (δ_H 3.05 and 3.70) of N60 1.8.12+13B in the COSY spectrum. Therefore the structure of compound N60 1.8.12+13B was determined as shown in Figure 3.15. A literature search found this compound to be identical to

prochaetoglobosin II, preciously isolated from a cell free system of *Chaetomium subaffine* under treatment with cytochrome P-450 inhibitors. The stereochemistry was decided by literature data comparison.[110]

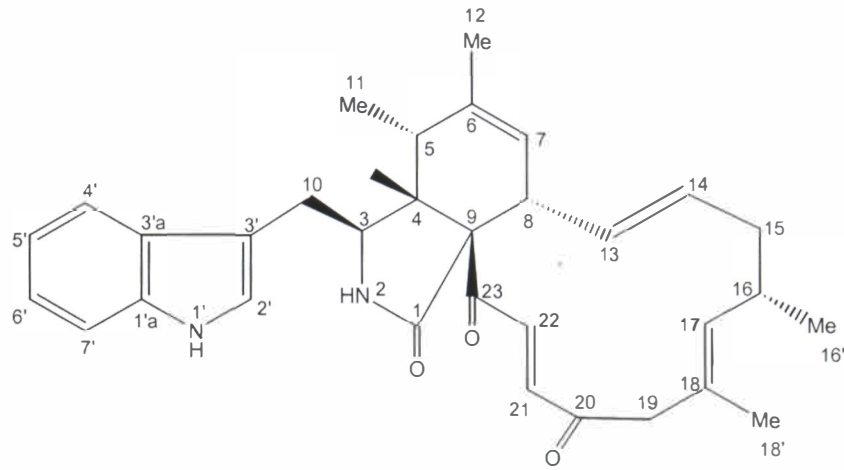


Figure 3.15 Structure of prochaetoglobosin II (N60 1.8.12+13B)

Table 3.3 ^{1}H NMR data[a] of chaetoglobosins (500 MHz in $CDCl_3$)

Proton atom	Chaetoglobosins				
	A	D	J	Pro I	Pro II
1'	8.32 (1H, s) (N-H)	8.26 (1H, br s) (N-H)	8.22 (1H, br s) (N-H)	8.16 (1H, br s) (N-H)	8.16 (1H, br s) (N-H)
2'	6.93 (1H, d, 2.5)	6.95 (1H, d, 2.0)	6.97 (1H, d, 2.0)	6.99 (1H, d, 2.0)	6.98 (1H, d, 2.0)
4'	7.48 (1H, d, 8.0)	7.46 (1H, d, 8.0)	7.49 (1H, d, 8.0)	7.51 (1H, d, 9.0)	7.51 (1H, d, 8.0)
5'	7.13 (1H, t, 7.5)	7.12 (1H, t, 7.5)	7.14 (1H, t, 6.5)	7.13 (1H, t, 7.5)	7.13 (1H, t, 7.5)
6'	7.19 (1H, t, 7.0)	7.19 (1H, t, 7.5)	7.21 (1H, t, 7.5)	7.20 (1H, t, 7.5)	7.20 (1H, t, 7.5)
7'	7.34 (1H, d, 8.0)	7.35 (1H, d, 8.0)	7.37 (1H, d, 8.5)	7.37 (1H, d, 8.0)	7.37 (1H, d, 8.0)
2	5.99 (1H, s) (N-H)	5.93 (1H, br s) (N-H)	5.68 (1H, br s) (N-H)	5.63 (1H, br s) (N-H)	5.62 (1H, br s) (N-H)
3	3.80 (1H, m)	3.48 (1H, m)	3.30 (1H, m)	3.30 (1H, m)	3.29 (1H, m)
4	3.01 (1H, dd, 5.0, 3.5)	3.01 (1H, t, 5.0)	3.12 (1H, m)	3.09 (1H, m)	3.14 (1H, m)
5	1.84 (1H, dd, 7.5, 5.5)	2.84 (1H, m)	2.47 (1H, m)	2.49 (1H, m)	2.47 (1H, m)
7	2.78 (1H, d, 5.0)	3.93 (1H, d, 10.5)	5.30 (1H, d, 1.5)	5.36 (1H, d, 1.5)	5.30 (1H, d, 1.5)
7-OH		1.84 (1H, s)			
8	2.12 (1H, dd, 9.5, 5.0)	2.31(1H, m)	2.53 (1H, m)	2.66 (1H, m)	2.55 (1H, m)
10a	H-10a: 2.94 (1H, dd, 14.5, 4.0)	H-10a: 3.07 (1H, m)	H-10a: 3.12 (1H, m)	H-10a: 3.02 (1H, m)	H-10a: 3.12 (1H, m)
10b	H-10b: 2.63 (1H, dd, 14.0, 8.0)	H-10b: 2.58 (1H, dd, 15.0, 9.5)	H-10b: 2.58 (1H, dd, 14.5, 10.0)	H-10b: 2.57 (1H, dd, 14.5, 10.0)	H-10b: 2.57 (1H, dd, 14.5, 10.0)
11 (5-CH_3)	1.24 (3H, d, 7.0)	1.33 (3H, d, 7.0)	1.43 (3H, d, 7.0)	1.33 (3H, d, 7.5)	1.42 (3H, d, 7.0)
12 (6-CH_3)	1.29 (3H, s)	5.44 (1H, br s)	1.80 (3H, s)	1.74 (3H, s)	1.79 (3H, s)
		5.21(1H, br s)			
13	6.05 (1H, dd, 15.0, 10.0)	5.87 (1H, dd, 14.5, 10.5)	5.91 (1H, dd, 15.0, 10.5)	6.10 (1H, dd, 15.0, 10.5)	5.90 (1H, dd, 15.0, 10.5)
14	5.20 (1H, m)	5.36 (1H, m)	5.17 (1H, m)	5.12 (1H, m)	5.17 (1H, m)
15a	2.25 (1H, br m)	2.29 (1H, m)	2.27 (1H, m)	2.27 (1H, m)	2.26 (1H, m)
15b	2.01 (1H, m)	2.06 (1H, m)	2.05 (1H, m)	1.93 (1H, m)	1.98 (1H, m)
16	2.43 (1H, br m)	2.47 (1H, br m)	2.47 (1H, br m)	2.43 (1H, br m)	2.46 (1H, br m)
16'	0.99 (3H, d, 7.0)	0.99 (3H, d, 6.5)	1.00 (3H, d, 6.0)	0.92 (3H, d, 6.0)	0.95 (3H, d, 7.0)
17	5.57 (1H, d, 9.5)	5.62 (1H, d, 8.0)	5.66 (1H, d, 8.5)	4.95 (1H, d, 8.0)	5.36 (1H, d, 8.0)
18'	1.31 (3H, s)	1.33 (3H, s)	1.35 (3H, s)	1.51 (3H, s)	1.48 (3H, s)
19	5.01 (1H, d, 4.5)	5.08 (1H, s)	5.10 (1H, s)	2.36 (1H, d, 13.0)	3.70 (1H, d, 13.0)
19a				2.13 (1H, d, 15.0)	3.05 (1H, d, 15.0)
19-OH	3.87 (1H, d, 4.5)		3.94 (1H, br s)		
20				2.38 (2H, m)	
21	6.45 (1H, d, 16.5)	6.56 (1H, d, 17.0)	6.59 (1H, d, 16.5)	6.79 (1H, d, 17.0)	6.44 (1H, d, 17.0)
22	7.71 (1H, d, 16.5)	8.02 (1H, d, 17.0)	8.23 (1H, d, 16.5)	7.07 (1H, d, 16.0)	8.18 (1H, d, 16.0)

[a] ^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by number of protons, multiplicity and coupling constant (J/Hz).

Table 3.4 ^{13}C NMR data (δ, ppm) of chaetoglobosins (recorded at 125 MHz in $CDCl_3$)

Carbon atom	Chaetoglobosins				
	A	**D**	**J**	**Pro I**	**Pro II**
1'a	136.6 (C)[a]	136.6 (C)	136.7 (C)	136.7 (C)	136.7 (C)
2'	122.6 (CH)	123.0 (CH)	122.9 (CH)	122.8 (CH)	122.9 (CH)
3'	110.3 (C)	111.4 (C)	111.7 (C)	111.9 (C)	111.8 (C)
3'a	127.2 (C)	127.0 (C)	127.0 (C)	127.0 (C)	127.0 (C)
4'	118.5 (CH)	118.7 (CH)	118.6 (CH)	118.6 (CH)	118.6 (CH)
5'	123.6 (CH)	120.0 (CH)	120.0 (CH)	120.0 (CH)	120.0 (CH)
6'	120.1 (CH)	122.7 (CH)	122.6 (CH)	122.5 (CH)	122.5 (CH)
7'	111.8 (CH)	111.7 (CH)	111.7 (CH)	111.7 (CH)	111.7 (CH)
1	173.4 (C)	172.1 (C)	172.8 (C)	172.8 (C)	172.8 (C)
3	52.8 (CH)	53.1 (CH)	54.2 (CH)	54.0 (CH)	54.0 (CH)
4	46.3 (CH)	46.1 (CH)	49.8 (CH)	50.2 (CH)	49.7 (CH)
5	36.3 (C)	32.7 (CH)	34.7 (C)	34.8 (CH)	34.7 (C)
6	58.3 (C)	148.7 (C)	141.2 (C)	139.9 (C)	141.0 (C)
7	62.6 (CH)	69.7 (CH)	125.8 (CH)	126.8 (CH)	126.0 (CH)
8	49.0 (CH)	50.6 (CH)	47.0 (CH)	47.0 (CH)	46.7 (CH)
9	63.5 (C)	62.4 (C)	66.3 (C)	66.3 (C)	66.3 (C)
10	34.4 (CH_2)	34.5 (CH_2)	34.9 (CH_2)	34.9 (CH_2)	34.7 (CH_2)
11 (5-CH_3)	13.7 (CH_3)	15.8 (CH_3)	15.0 (CH_3)	14.2 (CH_3)	14.9 (CH_3)
12 (6-CH_3)	20.1 (CH_3)	113.4 (CH_2)	20.5 (CH_3)	19.9 (CH_3)	20.3 (CH_3)
13	128.4 (CH)	128.2 (CH)	130.9 (CH)	129.6 (CH)	130.8 (CH)
14	133.9 (CH)	136.6 (CH)	132.2 (CH)	133.0 (CH)	132.2 (CH)
15	42.0 (CH_2)	42.1 (CH_2)	42.0 (CH_2)	41.2 (CH_2)	41.8 (CH_2)
16	32.3 (CH)	32.4 (CH)	32.5 (CH)	32.6 (CH)	32.3 (CH)
16' (16-CH_3)	21.2 (CH_3)	21.2 (CH_3)	21.1 (CH_3)	21.5 (CH_3)	21.1 (CH_3)
17	140.6 (CH)	140.4 (CH)	140.7 (CH)	132.9 (CH)	138.1 (CH)
18	132.5 (C)	132.9 (C)	132.5 (C)	130.8 (C)	128.7 (C)
18' (18-CH_3)	10.8 (CH_3)	10.8 (CH_3)	10.8 (CH_3)	16.5 (CH_3)	15.5 (CH_3)
19	81.9 (CH)	81.7 (CH)	81.5 (CH)	35.8 (CH_2)	53.2 (CH_2)
20	201.5 (C)	201.3 (C)	201.2 (C)	28.7 (CH_2)	201.3 (C)
21	131.7 (CH)	131.9 (CH)	131.3 (CH)	146.5 (CH)	134.4 (CH)
22	136.5 (CH)	137.0 (CH)	138.2 (CH)	128.3 (CH)	137.1 (CH)
23	196.7 (C)	196.9 (C)	197.8 (C)	198.3 (C)	198.3 (C)

[a] primary, secondary, tertiary and quaternary carbons, assigned by APT.

3.8.3 Structural elucidation of compound N60 1.6.7+8

Compound N60 1.6.7+8 was obtained as a pale yellow powder. The molecular formula of N60 1.6.7+8 was determined to be $C_{32}H_{38}O_6N_2$ after HRESMS analysis (found: 547.2805 [M+H$^+$], calcd: 547.2808), corresponding to a hydrated congener of chaetoglobosin D. The IR spectrum showed characteristic absorptions at 3060-3240 cm^{-1} (due to NH and OH groups), and 1695, 1685 cm^{-1} (due to carbonyl groups). Comparison of the ^{1}H and ^{13}C NMR spectra of N60 1.6.7+8 (Table 3.5) with those of chaetoglobosin D (Table 3.3, Table 3.4) showed good agreement except for differences at C-6. The olefinic carbon (δ_C 148.7, C-6) and the terminal methylene (δ_C 113.4, C-12, δ_H 5.21 and 5.44, H-12) in chaetoglobosin D were replaced by an oxygen-bearing aliphatic carbon (δ_C 76.1) and a methyl group (δ_C 22.3, δ_H 1.16). The assignment of C-6 in N60 1.6.7+8 was also supported by long-range correlations of H-7 (δ_H 3.54), H-11 (δ_H 1.32) and H-12 (δ_H 1.16) to C-6 (δ_C 76.1) (Figure 3.16). Two alcohol groups in the six membered ring suggested the 6, 7-diol structure in the cyclohexane part of N60 1.6.7+8. Thus, a planar structure as shown in Figure 3.16 was proposed for N60 1.6.7+8 and the structure confirmed by a series of 2D NMR experiments (COSY, HSQC and CIGAR, Table 3.5). N60 1.6.7+8 is a new compound and was named as chaetoglobosin Q. The 6, 7-diol in cyclohexane was a new type of structure in chaetoglobosins, but had been reported previously in cytochalasins P, Q and R.[112]

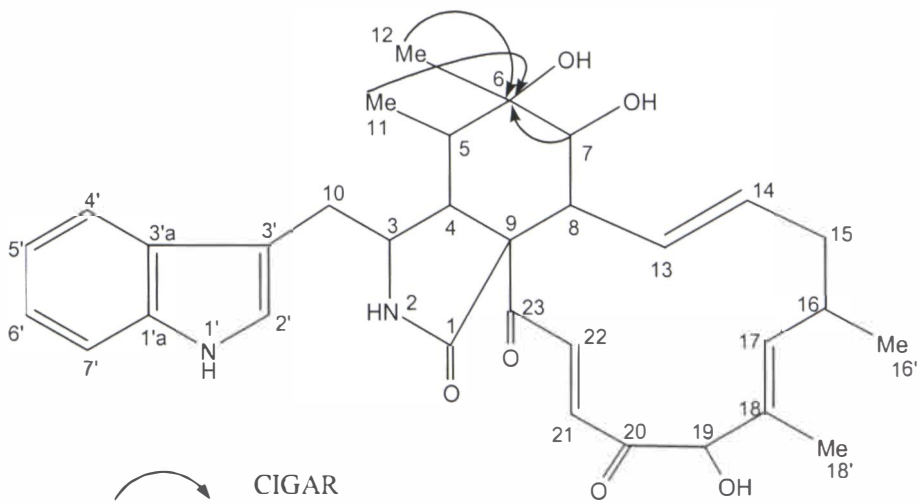


Figure 3.16 Planar structure of chaetoglobosin Q (N60 1.6.7+8)

The relative stereochemistry of chaetoglobosin Q (N60 1.6.7+8) was assigned by a NOESY spectrum (Table 3.5) and by reference to that described for chaetoglobosin A as determined by X-ray diffraction analysis.[79] The coupling constant ($J_{7\text{-}8}$ 11.5 Hz) showed the β configuration of the C-7 hydroxyl group. Since a positive NOE was observed from H-5 and H-8 to H-12, the methyl group of C-6 was suggested to be β while the C-6 hydroxy group was found to be in an α configuration. NOEs between H-3 to H-7 and H-11 suggested that these protons are in the α configuration, and NOEs from H-15a to H-16', from H-17 to H-16' and H-19, and from H-19 to H-17 and H-22 indicated these protons are also α. NOEs from H-15b to H-14 and from H-18' to H-16 implied that these protons are in the β configuration. Based on the evidence summarized above, the stereochemistry for chaetoglobosin Q (N60 1.6.7+8) was elucidated as depicted in Figure 3.17.

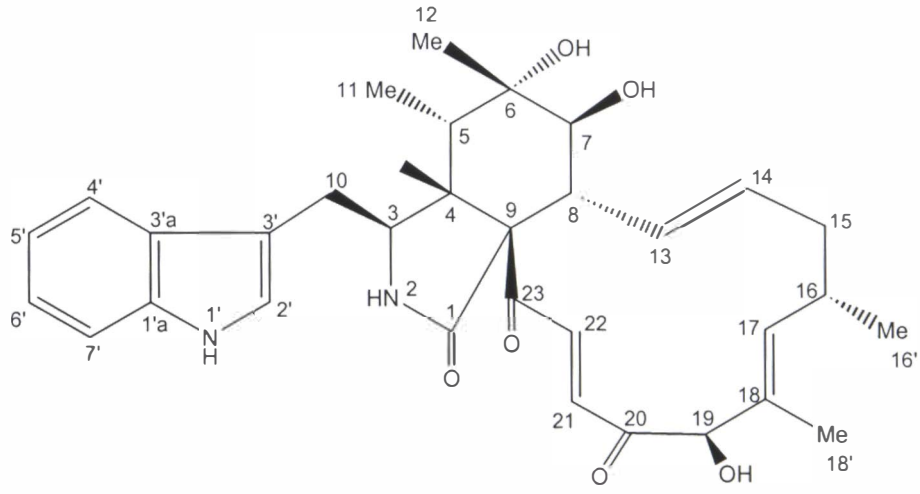


Figure 3.17 Structure of chaetoglobosin Q

Table 3.5 ^{1}H, ^{13}C, HSQC, COSY and CIGAR NMR data for N60 1.6.7+8[a]

no.	^{13}C δ[c]	^{1}H δ[b]	COSY	NOESY	CIGAR[d]
1'		8.34 (1H, br s) (N-H)	H-2'	H-2'	
1'a	136.6 (C)				
2'	122.9 (CH)	6.97 (1H, d, 2.0)	H-1'	H-1'	C-1'a, C-3', C-3'a
3'	111.9 (C)				
3'a	127.1 (C)				
4'	119.0 (CH)	7.58 (1H, d, 7.5)	H-5'	H-5', H-6'(w)	C-1'a, C-6'
5'	120.0 (CH)	7.10 (1H, t, 7.5)	H-4', H-6'	H-4', H-6'	C-3'a, C-7' (w)
6'	122.6 (CH)	7.18 (1H, t, 7.5)	H-5', H-7'	H-5', H-7'	C-1'a, C-4'
7'	111.6 (CH)	7.35 (1H, d, 8.5)	H-6'	H-6'	C-3'a, C-5'
1	171.9 (C)				
2		6.26 (1H, br s) (N-H)			C-4 (w), C-9
3	53.9 (CH)	4.19 (1H, m)	H-4, H-10b	H-7, H-11	
4	46.1 (CH)	2.78 (1H, dd, 7.0, 4.0)	H-3, H-5	H-11	
5	38.0 (CH)	1.92 (1H, dd, 7.5, 4.0)	H-4, H-11	H-4, H-11, H-12	
6	76.1 (C)				
7	74.7 (CH)	3.54 (1H, d, 11.5)	H-8	H-8, H-3	C-6, C-9
8	48.7 (CH)	2.04 (1H, m)	H-7, H-13	H-7, H-12	C-1, C-7, C-14
9	63.7 (C)				
10	35.1 (CH_2)	H-10a: 3.21 (1H, br d, 14.5)	H-3, H-10b	H-10b, H-11	C-2', C-3'
		H-10b: 2.47 (1H, dd, 14.5, 10.5)	H-3, H-10a	H-10a	C-2', C-3', C-3'a, C-3
11	13.4 (CH_3)	1.32 (3H, d,7.5)	H-5	H-3, H-4, H-5, H-10a	C-4, C-5, C-6
12	22.3 (CH_3)	1.16 (3H, s)		H-5, H-8	C-5, C-6, C-7
13	127.4 (CH)	5.67 (1H, dd, 15.0, 10.0)	H-8, H-14, H-15a(w)	H-14	
14	137.2 (CH)	5.32 (1H, m)	H-13, H-15a, H-15b	H-13, H-15b	
15	42.1 (CH_2)	H-15a: 2.32 (1H, m)	H-14, H-15b, H-16	H-16'(w)	
		H-15b: 2.05 (1H, m)	H-14, H-15a, H-16	H-14, H-15a	
16	32.5 (CH)	2.49 (1H, br m)	H-15b, H-16', H-17	H-16'	
16'	21.1 (CH_3)	0.99 (3H, d, 7.0)	H-16	H-15a, H-15b, H-16, H-17	C-15, C-16, C-17
17	140.5 (CH)	5.62 (1H, d, 9.5)	H-16, H-18'(w)	H-16', H-19	C-18', C-19
18	132.7 (C)				
18'	10.8 (CH_3)	1.34 (3H, s)	H-17	H-16	C-17, C-18, C-19
19	81.4 (CH)	5.09 (1H, s)		H-17, H-22(w)	C-17, C-18, C-18'
20	201.1 (C)				
21	132.0 (CH)	6.65 (1H, d, 17.0)	H-22	H-22	C-22(w), C-23
22	137.7 (CH)	8.22 (1H, d, 16.5)	H-21	H-17(w), H-19, H-21	C-20, C-23
23	197.1 (C)				

[a] ^{1}H NMR spectra recorded at 500 MHz in $CDCl_3$, ^{13}C NMR spectra recorded at 125 MHz in $CDCl_3$. [b] ^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by number of protons, multiplicity and coupling constant (J/Hz). [c] primary, secondary, tertiary and quaternary carbons, assigned by APT. [d] long range ^{1}H-^{13}C correlation from H to C observed in the CIGAR experiment.

3.8.4 Structural elucidation of compound N60 1511.4+5

Compound N60 1511.4+5 had the same molecular formula $C_{32}H_{38}O_6N_2$ as chaetoglobosin Q (N60 1.6.7+8), as deduced from HRESMS (found: 547.2796 [M+H$^+$], calcd: 547.2808). The ^{13}C NMR spectra of chaetoglobosin Q (Table 3.5) and N60 1511.4+5 (Table 3.6) were also similar except for slight differences at C-6, C-7, C-8, C-11 and C-12. From the molecular formula and the spectral data, N60 1511.4+5 was suspected to be an isomer in the cyclohexane moiety of chaetoglobosin Q. In the case of N60 1511.4+5, ^{1}H and ^{13}C NMR data including those obtained by COSY, HSQC and CIGAR (Table 3.6) revealed the connection from the C-10 to the C-5 methyl and from C-7 to the macrocyclic ring beyond C-8 as well as the quaternary carbon at C-6 bearing hydroxyl and methyl groups. The α configurations of H-7 and H-12 were determined from coupling constant ($J_{7\text{-}8}$ 10.5 Hz) and NOE interaction (H-7 to H-12) respectively. The structure shown in Figure 3.18 was proposed for N60 1511.4+5. This was proved to be the epimer of Q at the C-6 position.

N60 1511.4+5 is another new compound and was named chaetoglobosin R. As far as we know chaetoglobosin Q (N60 1.6.7+8) and R (N60 1511.4+5) are the only examples of chaetoglobosins with hydroxy substituents at both C-6 and C-7 positions.

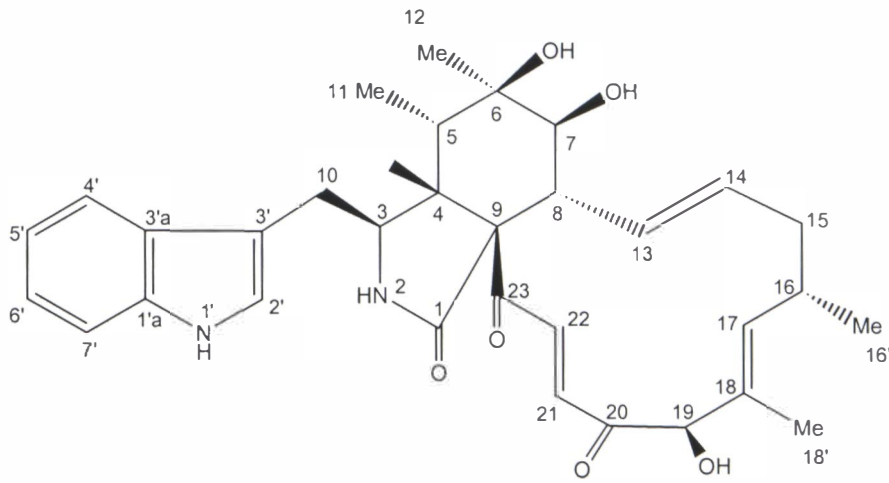


Figure 3.18 Structure of chaetoglobosin R (N60 1511.4+5)

Table 3.6 ^{1}H, ^{13}C, HSQC, COSY and CIGAR NMR data for N60 1511.4+5[a]

no.	^{13}C δ[c]	^{1}H δ[b]	COSY	NOESY	CIGAR[d]
1'		8.26 (1H, br s) (N-H)		H-2'	C-3', C-3'a
1'a	136.7 (C)				
2'	122.8 (CH)	7.00 (1H, d, 2.5)	H-1'	H-1', H-2(w), H-10b(w)	C-1'a, C-3', C-3'a
3'	111.7 (C)				
3'a	126.8 (C)				
4'	118.4 (CH)	7.50 (1H, d, 7.5)	H-5'	H-5', H-3, H-10a(w), H-11	C-1'a, C-3', C-3'a, C-6'
5'	120.2 (CH)	7.14 (1H, t, 7.5)	H-4', H-6'	H-4', H-6'	C-3'a, C-7'
6'	122.9 (CH)	7.22 (1H, t, 7.5)	H-5', H-7'	H-5', H-7'	C-1'a, C-4', C-7'
7'	111.8 (CH)	7.37 (1H, d, 8.5)	H-6'	H-6', H-1'	C-3'a, C-5', C-6'
1	171.6 (C)				
2		5.82 (1H, br s) (N-H)		H-3	C-3, C-4, C-9
3	53.8 (CH)	3.69 (1H, m)	H-4, H-10b	H-2, H-4', H-7, H-10a, H-10b, H-11, H-12	
4	45.0 (CH)	2.99 (1H, t, 4.5)	H-3, H-5	H-3(w), H-5, H-10a, H-10b, H-11	C-3, C-5(w), C-6, C-8, C-9, C-10, C-23
5	38.2 (CH)	2.24 (1H, m)	H-4, H-11	H-4, H-11, H-8	C-3, C-6, C-11
6	72.5 (C)				
7	71.4 (CH)	3.19 (1H, d, 10.5)	H-8	H-8, H-3, H-10b, H-12	
8	45.9 (CH)	2.38 (1H, t, 10.5)	H-7, H-13	H-5, H-7, H-14	C-1, C-6, C-7, C-13, C-14, C-23
9	63.2 (C)				
10	35.4 (CH_2)	H10a: 3.23 (1H, dd, 14.0, 11.5)	H-3, H-10b	H-3, H-10b, H-11	C-2', C-3'
		H10b: 2.52 (1H, dd, 14.5, 10.5)	H-3, H-4, H-10a	H-3, H-4, H-10a, H-2'(w)	C-2', C-3', C-3'a, C-3
11	15.2 (CH_3)	1.36 (3H, d, 8.5)	H-5	H-3, H-4, H-5, H-10a	C-4, C-5, C-6
12	25.1 (CH_3)	1.39 (3H, s)		H-3, H-7	C-5, C-6, C-7
13	127.7 (CH)	5.76 (1H, dd, 14.5, 10.5)	H-8, H-14	H-14, H-15b	C-15(w)
14	138.1 (CH)	5.41 (1H, m)	H-13, H-15a, H-15b	H-13, H-15a, H-16	
15	42.0 (CH_2)	H15a: 2.33 (1H, m)	H-14, H-15b, H-16	H-14, H-15b, H-16, H-16'	
		H15b: 2.06 (1H, m)	H-14, H-15a, H-16	H-13, H-15a, H-16(w), H-16', H-17(w)	C-13
16	32.5 (CH)	2.54 (1H, br m)	H-15b, H-16', H-17	H-14, H-15a, H-16', H-18'	
16'	21.1 (CH_3)	1.01 (3H, d, 4.5)	H-16	H-15a, H-15b, H-16, H-17	C-15, C-16, C-17
17	140.3 (CH)	5.63 (1H, d, 10.0)	H-16, H-18'(w)	H-15b, H-16', H-19, H-22	C-15(w), C-18', C-19
18	132.8 (C)				
18'	10.9 (CH_3)	1.35(3H, s)	H-17	H-16, H-19	C-17, C-18, C-19
19	81.4 (CH)	5.09 (1H, d, 3.5)	H-19' (19-OH)	H-17, H-22	
19'		3.95 (1H, d, 4.5)	H-19		
20	201.1 (C)				
21	132.5 (CH)	6.72 (1H, d, 16.5)	H-22	H-22	C-19, C-22, C-23
22	137.4 (CH)	8.17 (1H, d, 16.5)	H-21	H-21, H-17, H-19	C-20, C-23
23	196.0 (C)				

[a] ^{1}H NMR spectra recorded at 500 MHz in $CDCl_3$, ^{13}C NMR spectra recorded at 125 MHz in $CDCl_3$. [b] ^{1}H chemical shift values (δppm from $SiMe_4$) followed by number of protons, multiplicity and coupling constant (J/Hz). [c] primary, secondary, tertiary and quaternary carbons, assigned by APT. [d] long range ^{1}H-^{13}C correlation from H to C observed in the CIGAR experiment.

3.8.5 Structural elucidation of compound N60 178.8

Compound N60 178.8 had the molecular formula $C_{32}H_{38}O_3N_2$ by analysis of HRESMS (found: 499.2955 [M+H$^+$], calcd: 499.2961). The general features of its ^{1}H and ^{13}C NMR spectra (Table 3.7) closely resembled those of chaetoglobosin J (Table 3.3, Table 3.4) except for differences at C-20 (H-20) and C-19. The conjugated carbonyl signal at C-20 (δ_C 201.2) in chaetoglobosin J was replaced by a methylene signal (δ_C 35.4, δ_H 2.48) and the chemical shift of C-19 in N60 178.8, due to absence of the conjugated ketone group, shifted to a higher field (δ_C 76.6) than that found in chaetoglobosin J (δ_C 81.5). The assignment of C-20 (H-20) was also supported by the appearance of a doublet of triplets for H-21 (δ_H 6.80) in the ^{1}H NMR spectrum, and correlations between H-20 (δ_H 2.48) to H-21 and H-19 (δ_H 4.38) in the COSY spectrum (Table 3.7). Therefore, N60 178.8 was proposed as another reduced analogue of chaetoglobosin J. The structure was elucidated as shown in Figure 3.19 and this elucidation confirmed by a series of 2D NMR experiments (COSY, HSQC and CIGAR, Table 3.7). N60 178.8 was the third new compound and was named chaetoglobosin S.

The stereochemistry of N60 178.8 was deduced from NOESY spectral analysis (Table 3.7). The principal NOEs were: H-5 to H-8, H-3 to H-11, H-7 to H-12, H-8 to H-14, H-16 to H-18' and H-19 to H-17, H-22. These observations implied that the stereochemistry of N60 178.8 was the same as that of chaetoglobosin A (N60 1.7.4+5).[108,109]

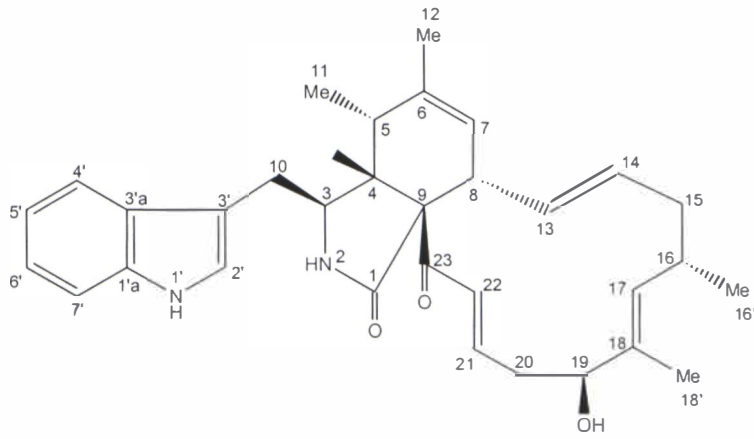


Figure 3.19 Structure of chaetoglobosin S (N60 178.8)

Table 3.7 ^{1}H, ^{13}C, HSQC, COSY and CIGAR NMR data for N60-178.8[a]

no.	^{13}C δ[c]	^{1}H δ[b]	COSY	NOESY	CIGAR[d]
1'		8.22 (1H, br s) (N-H)	H-2'	H-2'	
1'a	136.6 (C)				
2'	122.8 (CH)	6.99 (1H, d, 2.5)	H-1'	H-1'	C-1'a, C-3', C-3'a
3'	112.0 (C)				
3'a	127.0 (C)				
4'	118.7 (CH)	7.51 (1H, d, 7.5)	H-5'	H-5'	C-1'a, C-3'(w), C-6'
5'	119.9 (CH)	7.12 (1H, t, 7.5)	H-4', H-6'	H-4', H-6'	C-3'a, C-7' (w)
6'	122.5 (CH)	7.19 (1H, t, 7.5)	H-5', H-7'	H-5', H-7'	C-1'a, C-4'
7'	111.6 (CH)	7.36 (1H, d, 7.5)	H-6'	H-6'	C-3'a, C-5'
1	174.0 (C)				
2		5.67 (1H, br s) (N-H)		H-3	C-3(w), C-4 (w), C-9
3	54.0 (CH)	3.30 (1H, m)	H-2(w), H-4, H-10b	H-10a, H-10b(w), H-11	
4	50.7 (CH)	2.99 (1H, t, 5.0, 4.0)	H-2(w), H-3, H-5	H-5, H-11	C-3(w), C-5, C-6, C-8(w), 9(w), C-23
5	34.9 (CH)	2.50 (1H, dd, 7.5, 4.0)	H-4(w), H-11	H-4, H-8, H-11	
6	140.1 (C)				
7	126.6 (CH)	5.34 (1H, br s)	H-8, H-12	H-12	
8	46.7 (CH)	2.67 (1H, m)	H-7, H-12(w), H-13	H-5	
9	66.0 (C)				
10	35.0 (CH_2)	H10a: 3.03 (1H, dd, 14.0, 10.5)	H-3, H-10b	H-3, H-10b	
		H10b: 2.58 (1H, dd, 13.5, 10.0)	H-3, H-10a	H-10a	C-2', C-3', C-3'a, C-3
11	14.6 (CH_3)	1.34 (3H, d, 7.5)	H-5	H-3, H-4, H-5, H-12	C-4, C-5, C-6
12	20.3 (CH_3)	1.75 (3H, s)	H-7, H-8	H-7, H-11	C-5, C-6, C-7
13	130.3 (CH)	5.96 (1H, m)	H-8, H-14		
14	132.3 (CH)	5.10 (1H, m)	H-13, H-15b(w)	H-8	
15	41.3 (CH_2)	H15a: 2.21 (1H, m)	H-15b	H-15b, H-16'	
		H15b: 1.90 (1H, m)	H-14, H-15a	H-15a	
16	32.3 (CH)	2.47 (1H, br m)	H-16', H-17(w)	H-16', H-18'	
16'	21.4 (CH_3)	0.95 (3H, d, 7.0)	H-16	H-15a, H-16	C-15, C-16, C-17
17	137.1 (CH)	5.22 (1H, d, 8.0)	H-16, H-18'(w)	H-19	C-18', C-19
18	133.3 (C)				
18'	9.9 (CH_3)	1.52 (3H, d, 1.0)	H-17	H-16	C-17, C-18, C-19
19	76.9 (CH)	4.38 (1H, m)	H-20	H-17	C-17(w)
20	35.4 (CH_2)	2.48 (2H, m)	H-19, H-21		
21	142.5 (CH)	6.80 (1H, m)	H-20, H-22	H-20	C-23
22	128.4 (CH)	6.89 (1H, d, 16.0)	H-21	H-19	C-20(w), C-23(w)
23	197.7 (C)				

[a] ^{1}H NMR spectra recorded at 500 MHz in $CDCl_3$, ^{13}C NMR spectra recorded at 125 MHz in $CDCl_3$. [b] ^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by number of protons, multiplicity and coupling constant (J/Hz). [c] primary, secondary, tertiary and quaternary carbons, assigned by APT. [d] long range ^{1}H-^{13}C correlation from H to C observed in the CIGAR experiment.

Biological Activity:

The biological activities of chaetoglobosins isolated from *Chaetomium globosum* (N60) are listed in Table 3.8.

Table 3.8 The biological activity of chaetoglobosins

Sample	Cytotoxicity (IC_{50} ng/mL)					Antimicrobial Activity (mm inhibition)
	P388	A-549	HT-29	H-116	PC-3	
Chaetoglobosin A	1943	5000	>5000	>5000	>5000	Bs3, Cr5
Chaetoglobosin B	3447	>5000	>5000	5000	>5000	Bs1
Chaetoglobosin D	4919	>5000	5000	>5000	>5000	Bs3
Chaetoglobosin J	2469	>5000	>5000	>5000	>5000	Bs2
Chaetoglobosin Q	-	>5000	>5000	>5000	>5000	-
Chaetoglobosin R	2190	>5000	>5000	5000	>5000	-
Chaetoglobosin S	1575	5000	5000	2500	5000	-
Prochaetoglobosin I	3988					Tm2
Prochaetoglobosin II	3988					Tm2

P388 = Murine leukaemia
A-549 = Human lung carcinoma
HT-29 = Human colon carcinoma
H-116 = Human colon carcinoma
PC-3 = Human prostate carcinoma
Bs = *Bacillus subtilis*
Cr = *Cladosporium resinae*
Tm = *Trichophyton mentagrophytes*
BsX (or CrX): X mm inhibition at a concentration of 15 μg/disk.
TmX: X mm inhibition at a concentration of 30 μg/disk.
- none detected

3.8.6 Structural elucidation of compound N60 1.7.3

The molecular formula of compound N60 1.7.3 was determined to be $C_{23}H_{29}ClO_7$ based on analysis of HRESMS (found 433.1429 [M-H^+-H_2O], calcd: 433.1418) and ^{13}C NMR (Table 3.9). The ^1H NMR and HSQC spectra (Table 3.9) disclosed the presence of one primary methyl group (δ_H 0.89, H-13), three secondary methyl groups (δ_H 1.06, 11-CH_3; δ_H 1.12, 4'-CH_3; δ_H 1.39, H-6'), one tertiary methyl group (δ_H 1.39, 7-CH_3), one methylene group (δ_H 1.41, H-12), five methine groups (δ_H 1.89, H-4'; δ_H 2.25, H-11; δ_H 2.98, H-8; δ_H 3.06, H-2' and δ_H 4.30, H-5') including one oxygen-bearing methine (δ_C 77.2, C-5'), two trisubstituted olefinic protons (δ_H 6.54, H-4; δ_H 7.27, H-1) and one *trans*-olefinic group (δ_H 6.05, H-9; δ_H 6.51, H-10, J=16.0 Hz). The identity of the remaining groups was revealed by analysis of the ^{13}C NMR (APT) spectra: two carbonyl carbons (δ_C 170.8, C-1'; δ_C 189.5, C-6); four olefinic carbons (δ_C 110.2, C-5; δ_C 114.5, C-8a; δ_C 140.7, C-4a and δ_C 157.9, C-3) and two oxygen-bearing quaternary carbons (δ_C 84.1, C-7 and δ_C 104.4, C-3').

The analysis of the COSY spectrum (Table 3.9) led to the partial structural units as shown by bold-faced lines in Figure 3.20.

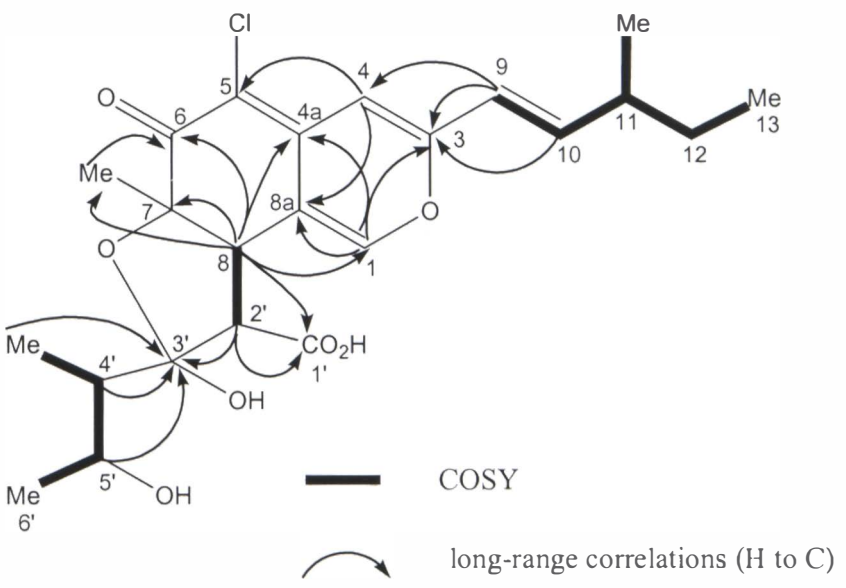


Figure 3.20 Structure of chaetoviridin B (N60 1.7.3)

The connection of functional groups was demonstrated on the basis of long-range

correlations observed in the CIGAR experiment (Table 3.9, Figure 3.20). The long-range correlations of H-9 to C-3 and C-4 and H-10 to C-3 led to connection of C-9 to C-4 (δ_C 105.2) through C-3. The long-range correlations of H-1 to C-3, C-4a, C-8a, H-4 to C-8a and the chemical shifts of C-1 and C-3 (δ_C 145.8 and 157.9, respectively) allow the connection of C-1 to C-3 through an oxygen and C-1 to C-4 through C-8a and C-4a. The long-range correlations of H-8 to C-7, C-6, C-1, C-4a and to 7-CH_3 carbon (δ_C 23.5) and correlations between the protons of 7-CH_3 to C-6 resulted in connection of C-6 to C-8a through C-7 and C-8 and the assignment of 7-CH_3. Connection of C-4a to C-6 through C-5 was deduced by long-range correlations between H-4 to C-5 and the chemical shift of C-6 (δ_C 189.5, a conjugated carbonyl carbon). Connection of C-2' (δ_C 58.5) to C-4' (δ_C 45.2) through C-3' (δ_C 104.4) was indicated by the long-range correlations of H-2', H-4', H-5' and the protons of 4'-CH_3 to C-3'. Connection of C-1' to C-2' was determined by the long-range correlations between H-2' and H-8 to C-1'. The appearance of a chlorine atom in the molecule was suggested by the isotope peaks in the mass spectrum. The connection of C-7 to C-3' through oxygen and all the other assignments were made by analysis of chemical shifts and the molecular formula. Finally, the structure as shown in Figure 3.20 was proposed for compound N60 1.7.3. After a literature search, this compound was found to be identical to chaetoviridin B, previously isolated from the culture of *Chaetomium globosum* var. *flavo-viridae*.[113]

Biological Activity:

P388 activity: IC_{50} >12500 ng/mL

Antimicrobial activity: none detected

Table 3.9 ^{1}H, ^{13}C, HSQC, COSY and CIGAR NMR data for compound N60 1.7.3[a]

no	^{13}C δ[c]	^{1}H δ[b]	COSY	CIGAR[d]
1	145.8 (CH)	7.27 (1H, s)		C-3, C-4a, C-8, C-8a
3	157.9 (C)			
4	105.2 (CH)	6.54 (1H, s)		C-3, C-5, C-8a, C-9
4a	140.7 (C)			
5	110.2 (C)			
6	189.5 (C)			
7	84.1 (C)			
7-CH_3	23.5 (CH_3)	1.39 (3H, s)		C-6, C-7, C-8
8	50.7 (CH)	2.98 (1H, d, 9.5)	H-2'	C-1, C-4a, C-6, C-7, 7-CH_3, C-8a, C-1', C-2'
8a	114.5 (C)			
9	120.4 (CH)	6.05 (1H, d, 16.0)	H-10	C-3, C-4, C-11
10	147.1 (CH)	6.51 (1H, dd, 15.5, 7.5)	H-9, H-11	C-3, C-11, 11-CH_3, C-12
11	39.1 (CH)	2.25 (1H, m)	H-10(w), 11-CH_3, H-12	C-9, C-10, 11-CH_3, C-12(w), C-13(w)
11-CH_3	19.6 (CH_3)	1.06 (3H, d, 7.0)	H-11	C-10, C-11, C-12
12	29.4 (CH_2)	1.41 (2H, m)	H-11, H-13	C-10, C-11, 11-CH_3, C-13
13	11.9 (CH_3)	0.89 (3H, t, 7.5)	H-12	C-11, C-12
1'	170.8 (C)			
2'	58.5 (CH)	3.06 (1H, d, 9.5)	H-8	C-8, C-8a, C-1', C-3'
3'	104.4 (C)			
4'	45.2 (CH)	1.89 (1H, m)	4'-CH_3, H-5'	C-3', 4'-CH_3, C-5'
4'-CH_3	9.0 (CH_3)	1.12 (3H, d, 6.5)	H-4'	C-3', C-4', C-5'
5'	77.2 (CH)	4.30 (1H, m)	H-4', H-6'	C-3'
6'	18.9 (CH_3)	1.39 (3H, d, 6.0)	H-5'	C-4', C-5'

[a] ^{1}H NMR spectra recorded at 500 MHz in $CDCl_3$, ^{13}C NMR spectra recorded at 126 MHz in $CDCl_3$.
[b] ^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by number of protons, multiplicity and coupling constant (J/Hz).
[c] primary, secondary, tertiary and quaternary carbons, assigned by APT.
[d] long range ^{1}H-^{13}C correlation from H to C observed in the CIGAR experiment.

3.8.7 Structural elucidation of compound N60 1.7.2A

The ^{1}H and ^{13}C NMR spectra (Table 3.10) of compound N60 1.7.2A were similar to those of chaetoviridin B (N60 1.7.3, Table 3.9) except for the differences in C-8 (H-8), C-2' (H-2') and C-3'. Two methine groups at δ_C 50.7 (C-8) and δ_C 58.5 (C-2') in chaetoviridin B were replaced by two olefinic carbons (δ_C 163.1 and 125.1, respectively) in N60 1.7.2A. The oxygen-bearing quaternary carbon at C-3' (δ_C 104.4) in chaetoviridin B was absent and instead a conjugated carbonyl (δ_C 201.1) observed in N60 1.7.2A. These observations, along with the molecular formula ($C_{23}H_{25}ClO_6$), were accommodated by the structure as shown in Figure 3.21. This compound was identical to chaetoviridin A, isolated previously together with chaetoviridin B from the culture of *Chaetomium globosum* var. *flavo-viridae*.[113]

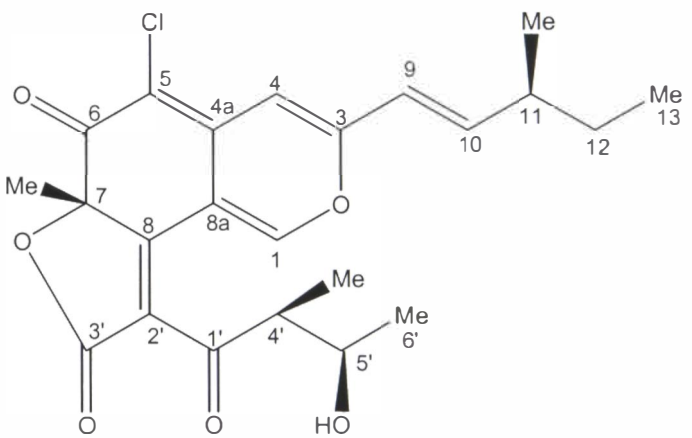


Figure 3.21 Structure of chaetoviridin A (N60 1.7.2A)

Biological Activity:

P388 activity: IC_{50} >12500 ng/mL

Antimicrobial activity: none detected

Chaetoviridin A has been reported to exhibit a weak inhibitory activity on monoamine oxidase (IC_{50} 1.2 x 10^{-2} g/mL), an induction of chlamydospore-like cells (40-50% at 100 μg/disc), and an inhibition of growth of *Pyricuralia oryzae* (2.5 μg/mL).[113]

Table 3.10 ^{1}H and ^{13}C NMR data for compound N60 1.7.2A[a]

no	^{13}C δ^{c}	^{1}H δ^{b}	no	^{13}C δ^{c}	^{1}H δ^{b}
1	151.8 (CH)	8.77 (1H, s)	11	39.2 (CH)	2.28 (1H, m)
3	157.2 (C)		11-CH_3	19.5 (CH_3)	1.09 (3H, d, 6.5)
4	105.6 (CH)	6.54 (1H, s)	12	29.4 (CH_2)	1.41 (2H, m)
4a	139.8 (C)		13	11.9 (CH_3)	0.90 (3H, t, 7.0)
5	109.1 (C)		1'	168.1 (C)	
6	183.5 (C)		2'	125.1 (C)	
7	87.8 (C)		3'	201.1 (C)	
7-CH_3	26.5 (CH_3)	1.69 (3H, s)	4'	51.1 (CH)	3.63 (1H, m)
8	163.1 (C)		4'-CH_3	21.7 (CH_3)	1.17 (3H, d, 7.0)
8a	110.6 (C)		5'	71.1 (CH)	3.86 (1H, m)
9	119.9 (CH)	6.07 (1H, d, 15.5)	6'	13.8 (CH_3)	1.16 (3H, d, 6.0)
10	148.3 (CH)	6.59(1H, dd, 16.0, 8.0)			

[a] ^{1}H NMR spectra recorded at 500 MHz in $CDCl_3$, ^{13}C NMR spectra recorded at 126 MHz in $CDCl_3$.
[b] ^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by number of protons, multiplicity and coupling constant (J/Hz).
[c] primary, secondary, tertiary and quaternary carbons, assigned by APT.

3.8.8 Structural elucidation of compound N60 1910.1a.22-25

Compound N60 1910.1a.22-25 was isolated as a crystalline compound. The molecular formula was determined to be $C_{28}H_{44}O_3$ by analysis of the ^{13}C NMR and ^{1}H NMR data (Table 3.11). Ions in the EIMS spectrum *m/z* 428, 410 and 396 correspond to $[M]^+$, $[M\text{-}H_2O]^+$ and $[M\text{-}O_2]^+$, respectively. The ^{1}H NMR spectrum showed signals due to two tertiary methyl groups (δ_H 0.80, H-18; δ_H 0.87, H-19) and four secondary methyl groups (δ_H 0.81, H-26; δ_H 0.82, H-27; δ_H 0.90, H-28 and δ_H 0.99, H-21), which suggested an ergostane skeleton.[114] Other signals in the ^{1}H NMR spectrum disclosed an oxygenated methine proton (δ_H 3.96, H-3) and four disubstituted olefinic protons (δ_H 5.13, H-22; δ_H 5.21, H-23; δ_H 6.24, H-6 and δ_H 6.50, H-7). The identity of the remaining groups was indicated by HSQC and APT spectra (Table 3.11): seven methylene groups (δ_C 20.8, C-15; δ_C 23.6, C-11; δ_C 28.8, C-16; δ_C 30.3, C-2; δ_C 34.9,

C-1; δ_C 37.1, C-4 and δ_C 39.5, C-12); six methine groups (δ_C 33.2, C-25; δ_C 39.9, C-20; δ_C 42.9, C-24; δ_C 51.2, C-9; δ_C 51.8, C-14 and δ_C 56.4, C-17) and four quaternary carbons (δ_C 36.3, C-10; δ_C 44.7, C-13; δ_C 79.6, C-8 and δ_C 82.3, C-5). The chemical shifts of C-5 and C-8 suggested the presence of a peroxy group.[115]

A close inspection of the ^{1}H and ^{13}C NMR spectra of N60 1910.1a.22-25 by COSY and HSQC experiments (Table 3.11) led to the assignments of partial structural units as shown by bold lines in Figure 3.22. These assignments were confirmed by long-range correlations observed in the CIGAR experiment (Table 3.11).

The assignments of the remaining groups were based mainly on the CIGAR data (Table 3.11, Figure 3.22). The principle correlations were as follows: H-18 to C-12, C-13, C-14 and C-17; H-19 to C-1, C-5 and C-9; H-4 to C-5; H-6 to C-4, C-5 and C-8; H-7 to C-5, C-8 and C-9 and H-15 to C-8 and C-13.

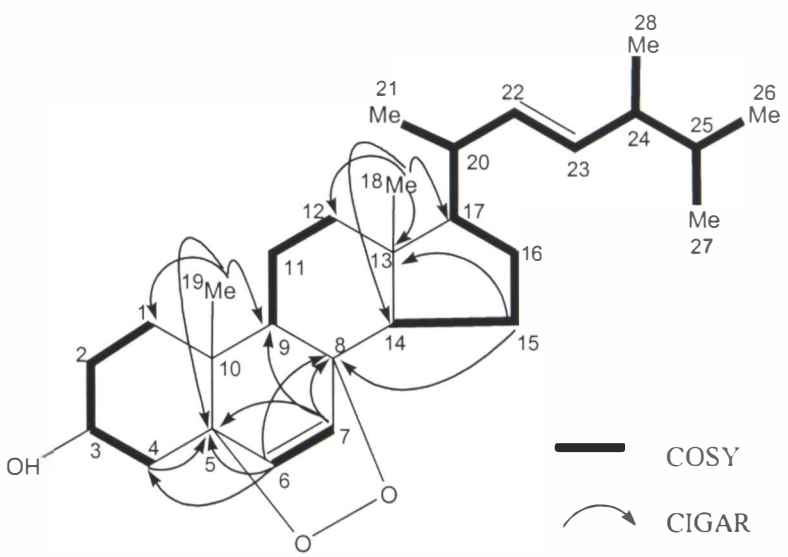


Figure 3.22 Planar structure of N60 1910.1a.22-25

The planar structure as depicted in Figure 3.22 was proposed for compound N60 1910.1a.22-25. The stereochemistry of N60 1910.1a.22-25 was confirmed to be 5α,8α-epidioxyergosta-6,22-dien-3β-ol (ergosterol peroxide) (Figure 3.23) by comparing its spectral data (MS, ^{1}H NMR, ^{13}C NMR) with that reported in the literature.[116-119]

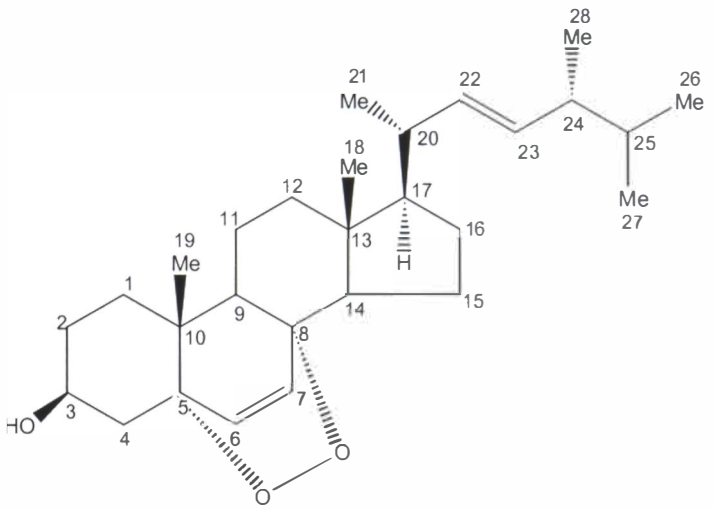


Figure 3.23 Structure of ergosterol peroxide (N60 1910.1a.22-25)

Biological Activity:

P388 activity: IC_{50} 8469 ng/mL

Antimicrobial activity: none detected

This is the first report of the production of ergosterol peroxide by a *Chaetomium* sp. although it has been reported from a wide variety of fungi, lichens, sponges and some marine organisms.[118] It has been suggested to be an important intermediary in the biosynthesis of ergosterol.[117] Ergosterol peroxide is reported to have antitumour, antiviral, antioxidant and anticomplementary activity, and displays potent antitumour activity against Walker 256 carcinosarcoma and MCF-7 breast cancer cell lines.[118] In the antiviral test, all 4 influenza A virus strains on chick chorioallantoic membrane were inhibited in a concentration-dependent manner by 10-40 μg ergosterol peroxide/mL.[120] Ergosterol peroxide shows potent inhibition of lipid peroxidation and exhibits higher antioxidant activity than the well-known antioxidants, ax-tocopherol and thiourea.[116] It also shows very strong anticomplementary activity on the classical pathway, the IC_{50} values being 5.0 μM and 1.0 μM, respectively.[121]

Table 3.11 ^{1}H, ^{13}C, HSQC, COSY and CIGAR NMR data for N60-1910.1a.22-25[a]

no.	^{13}C δ[c]	^{1}H δ[b]	COSY	CIGAR[d]
1	34.9 (CH_2)	1.94 (1H, m)	1.69, 1.52	18.3, 51.2
		1.69 (1H, m)	1.94	
2	30.3 (CH_2)	1.52 (1H, m)	3.96, 1.84, 1.94	
		1.84 (1H, m)	1.52, 3.96	
3	66.6 (CH)	3.96 (1H, m)	2.10, 1.90, 1.52, 1.84	
4	37.1 (CH_2)	2.10 (1H, m)	3.96, 1.90	82.3, 66.6, 30.3
		1.90 (1H, m)	3.96, 2.10	66.6, 30.3(w)
5	82.3 (C)			
6	135.6 (CH)	6.24 (1H, d, 8.5)	6.50	79.6, 82.3, 37.1
7	130.9 (CH)	6.50 (1H, d, 9.0)	6.24	79.6, 82.3, 51.2
8	79.6 (C)			
9	51.2 (CH)	1.49(1H, m)	1.21	79.6, 23.6, 130.9, 34.9
10	36.3 (C)			
11	23.6 (CH_2)	1.49 (1H, m)	1.21, 1.94	79.6, 44.7
		1.21 (1H, m)	1.94, 1.49	44.7
12	39.5 (CH_2)	1.94 (1H, m)	1.21, 1.49	44.7, 51.2, 51.8
		1.21 (1H, m)	1.94, 1.49	44.7
13	44.7 (C)			
14	51.8 (CH)	1.55 (1H, m)	1.38	79.6, 44.7, 130.9, 13.0
15	20.8 (CH_2)	1.58 (1H, m)	1.38, 1.74	79.6, 44.7
		1.38 (1H, m)	1.74	
16	28.8 (CH_2)	1.74 (1H, m)	1.21, 1.34, 1.38, 1.58	
		1.34 (1H, m)	1.21, 1.58, 1.74	
17	56.4 (CH)	1.21(1H, m)	1.74	44.7, 39.9
18	13.0 (CH_3)	0.80 (3H, s)		56.4, 51.8, 44.7, 39.5
19	18.3 (CH_3)	0.87 (3H, s)		51.2, 34.9, 37.1, 82.3, 23.6(w)
20	39.9 (CH)	2.00 (1H, m)	0.99, 5.13, 1.21	135.4, 132.5, 56.4
21	21.0 (CH_3)	0.99 (3H, d, 7.0)	2.00	39.9, 56.4, 135.4
22	135.4 (CH)	5.13 (1H, dd, 15.0, 7.5)	5.21, 2.00	42.9, 39.9(w), 132.5(w)
23	132.5 (CH)	5.21 (1H, dd, 15.0, 8.5)	5.13, 1.84	42.9(w), 39.9 135.4(w)
24	42.9 (CH)	1.84 (1H, m)	5.21, 0.90, 1.45	132.5, 135.4, 33.2, 17.7
25	33.2 (CH)	1.45 (1H, m)	0.81, 0.82, 1.84	42.9, 20.1(w), 17.7(vw), 132.5(w)
26	19.8 (CH_3)	0.81(3H, d, 8.0)	1.45	42.9, 33.2
27	20.1 (CH_3)	0.82 (3H, d, 7.5)	1.45	19.8, 33.2, 42.9
28	17.7 (CH_3)	0.90 (3H, d, 7.0)	1.84	33.2, 42.9, 132.5

[a] ^{1}H NMR spectra recorded at 500 MHz in $CDCl_3$. ^{13}C NMR spectra recorded at 126 MHz in $CDCl_3$. [b] ^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by number of protons, multiplicity and coupling constant (J/Hz). [c] pimary, secondary, tertiary and quaternary carbons, assigned by APT. [d] long range ^{1}H-^{13}C correlation from H to C observed in the CIGAR experiment.

3.9 Discussion

Bioassay- and ^{1}H NMR-guided fractionation of the EtOAc extract of *Chaetomium globosum* (N60) by C_{18} column chromatography, followed by normal phase chromatography (Diol column), afforded twelve compounds. These compounds belong to three structural classes: cytochalasins, azaphilones and steroids. On the basis of the molecular formulae determined by high resolution MS and the ^{1}H and ^{13}C NMR data, it was determined that nine of the compounds had been previously characterised and were: chaetoglobosins A, B, D, J, prochaetoglobosins I and II, chaetoviridins A and B, and ergosterol peroxide. Prochaetoglobosins I and II were produced previously by treatment of *Chaetomium subaffine* with specific cytochrome P-450 inhibitors by Oikawa[110] and hence this is the first report of naturally occurring prochaetoglobosins I and II. Ergosterol peroxide has been isolated from several fungi previously but has not been recorded from *Chaetomium* spp. Three new members of the chaetoglobosin family were isolated and were named chaetoglobosins Q, R and S. In the assay against the P388 murine leukaemia cell line, most of the chaetoglobosins exhibited significant activity (IC_{50} 1943-3988 ng/mL). Most also showed antibacterial or antifungal activities.

Chapter 4

PHOMA SP. (K31)

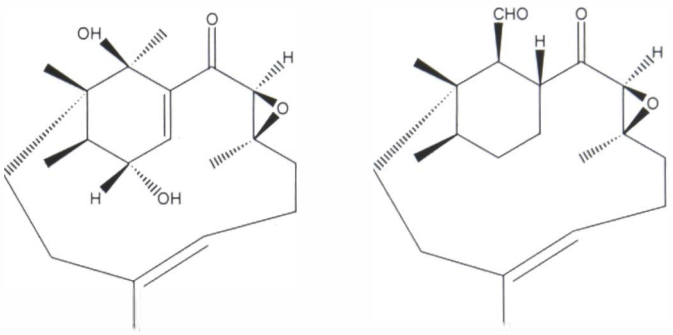
4.1 Introduction

Phoma is a large and variable genus with more than 2000 species described.[122] The genus is characterised by the production of black subsurface pycnidia which extrude small, single-celled conidia in slime. Some species are plant pathogens, for example, *Phoma exigua var populi var. nov.* causes necrotic lesions on poplars,[123] *Phoma gentianae-sino-ornatae sp. nov.* causes root rot in *Gentiana sino-ornata* plants.[124] Although *Phoma* is a large genus, the literature on the bioactive metabolites isolated from this genus is relatively small and only 98 compounds have been recorded on Antibase.[75] Several important metabolites produced by *Phoma* species will be discussed in this chapter.

Phomactins

Phomactins are diterpenes first isolated by Sugano and co-workers in their screening for platelet activating factor (PAF) antagonists from marine fungi.[125] PAF causes platelet aggregation, chemotaxis and degranulation of polymorphonuclear leukocytes, smooth muscle contraction, vascular permeability and hypotension. PAF may be involved in many inflammatory, respiratory, and cardiovascular diseases. So far nine phomactins (phomactins A, B, B_1, B_2, C-G) have been isolated from a culture broth of a marine *Phoma* sp. (SANK 11486) and most antagonise PAF action.[126,127] Phomactin D (Figure 4.1) is the most potent PAF antagonist of the phomactin family and inhibits the binding of PAF to its receptors and PAF-induced platelet aggregation with IC_{50}

values of 2.8 x 10^{-7} M and 8.0 x 10^{-7} M respectively.[126] Phomactin B (Figure 4.1) is thought to have potential for treatment of asthma, peptic ulcers, renal disorders and hypertension.[128]

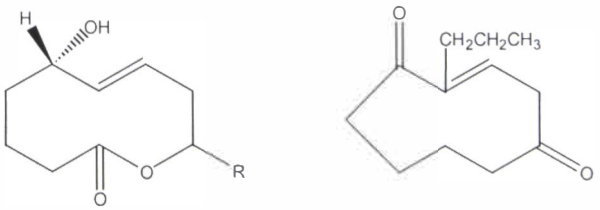


Phomactin B Phomactin D

Figure 4.1 Structures of phomactins B and D

Putaminoxins

Putaminoxins are metabolites produced by the fungus *Phoma putaminum*, the pathogen of leaf necrosis of *Erigeron annuus*, a common weed of field and pasture. When assayed on leaves of host and non-host plants, putaminoxin and putaminoxin C (Figure 4.2) showed toxic effects[129] while putaminoxin B (Figure 4.2), D and E showed no phytotoxicity.[130,131] Considering the semiselective phytotoxic activity against the host plant, putaminoxin and putaminoxin C could be used as herbicides or as analogues for the development of selective and safe herbicides.



R Putaminoxin C

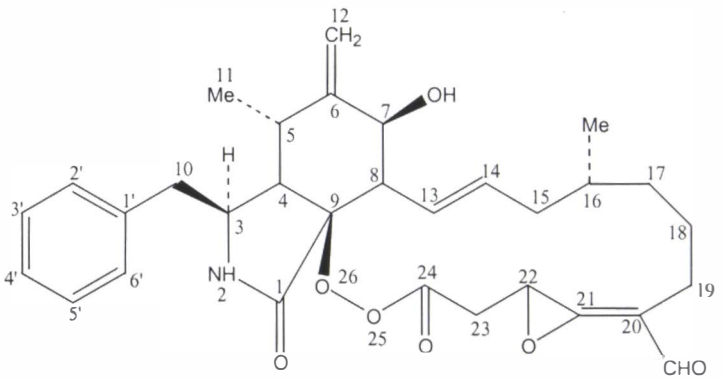
Putaminoxin $CH_2CH_2CH_3$

Putaminoxin B $CH_2CH_2CH_2CH_2CH_3$

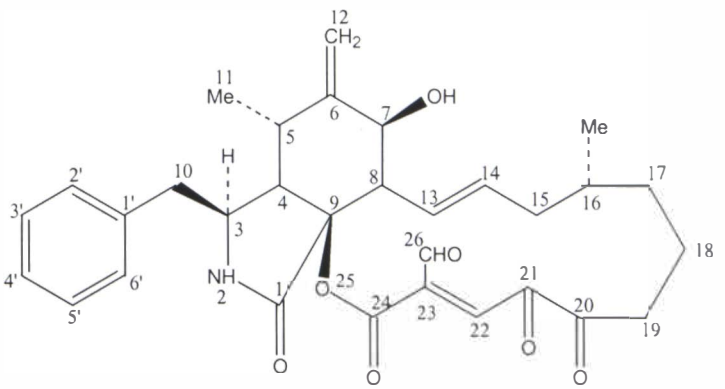
Figure 4.2 Structures of putaminoxins

Cytochalasins

Several cytochalasins have been isolated from the filtrate of *Phoma exigua var. heteromorpha*, the fungal causal agent of a foliar disease of Oleander (*Nerium oleander* L.). Besides known cytochalasins such as cytochalasins A, B, F, deoxaphomin and ascochalasin, four new cytochalasins, cytochalasins T,[132] U, V[133] and W[134] have also been isolated from this fungus. Cytochalasins U and V (Figure 4.3) represent the first natural occurrence of a 25, 26-dioxa [16] and 25-oxa [15] cytochalasan, respectively. Most of the cytochalasins isolated from *P. exigua var. heteromorpha* showed toxic activity against the larvae of the brine shrimp (*Artemia salina*).



Cytochalasin U



Cytochalasin V

Figure 4.3 Structures of cytochalasins U and V

4.2 Culturing and Isolation of Metabolites

4.2.1 Culturing and extraction

Isolate K31 belonging to the genus *Phoma* (Figure 4.4) was collected from a driftwood sample in Vancouver, British Colombia (BC), Canada.

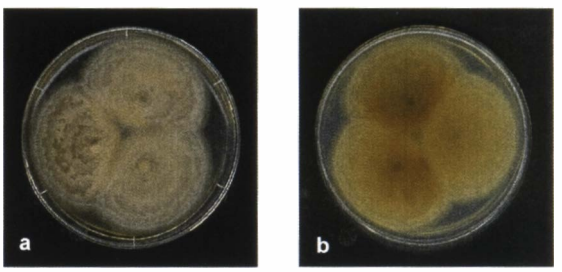


Figure 4.4 Colonies of *Phoma* sp. (K31) on PDA (6 days) (a) top; (b) reverse

K31 was cultured on PDA Petri dishes for eight days at 25 °C. The mycelial discs were then cut from the growing margin of the colony and inoculated into ½ PDB. Two batches of the broth were cultured at 26 °C in static conditions. A 'big broth' I was cultured for four weeks and the 'big broth' II was cultured for eleven weeks. Each culture was filtered under suction and the mycelium and filtrate were each extracted separately with EtOAc. EtOAc extracts were evaporated under reduced pressure and then assayed for activity against the P388 cell line. Among the four extracts assayed, only the filtrate extract from 'big broth' I showed moderate activity (Table 4.1). Since no P388 activity was detected from the extract of 'big broth' II, it was assumed that any active compounds might have decomposed or been metabolised or converted into inactive compounds during the long period of culture. The active extract from 'big broth' I was selected for further purification.

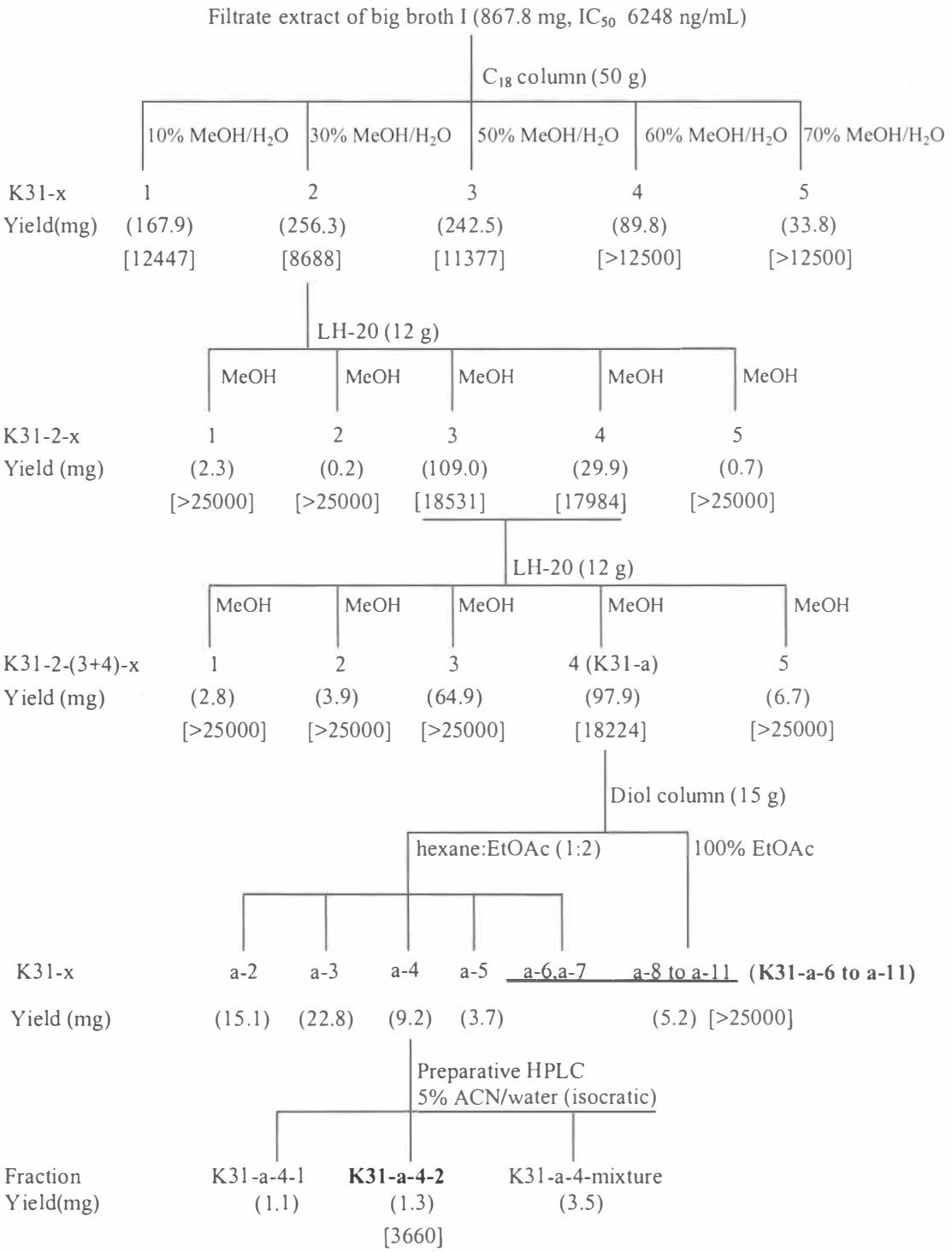
Table 4.1 Yield and activity of extracts from *Phoma* sp. (K31)

	'Big Broth' I (6 L, 4 weeks)		**'Big Broth' II (2 L, 11 weeks)**	
	Filtrate Extract	Mycelial Extract	Filtrate Extract	Mycelial Extract
Yield	867.8 mg	1632.5 mg	392.5 mg	467.7 mg
P388 activity (IC_{50})	6248 ng/mL	45141 ng/mL	> 62500 ng/mL	22499 ng/mL

4.2.2 Chromatography of Phoma sp. (K31)

An outline of the isolation is given in **Scheme 4.1**.

Scheme 4.1



[ ]: P388 activity (IC_{50} = ng/mL)

The filtrate extract (867.8 mg) from the big broth I of *Phoma* sp. (K31) was separated on a C_{18} column, eluted with a stepped gradient from 10% MeOH/H_2O to MeOH and then to DCM. Fourteen fractions were collected and combination was based mainly on TLC. Both mass and P388 activity were concentrated in the first three fractions (Scheme 4.1), suggesting the active compounds were polar. Among the three active fractions, fraction K31-2 showed moderate activity (IC_{50} 8688 ng/mL) while fractions K31-1 and K31-3 showed very weak activity (IC_{50} >10, 000 ng/mL).

Fraction K31-2 (256.3 mg) was applied to a LH-20 column (12 g) and washed with straight MeOH. Fourteen fractions were collected. Bioassay analysis of all eluted fractions only recovered very weak activity in the fractions K31-2-3 and K31-2-4 (Scheme 4.1). The possible reasons for the activity decreasing after purification is the decomposition of active compounds during chromatography or the active compounds being retained on the columns.

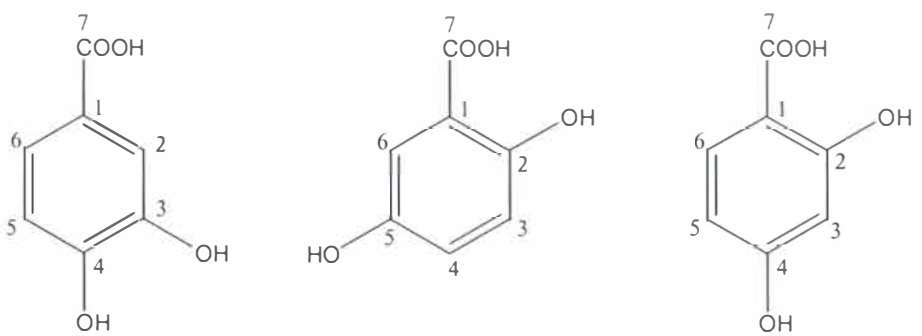
Fractions K31-2-3 (109.0 mg) and K31-2-4 (29.9 mg) were combined based on the HPLC profiles and purified on another LH-20 column. The column was eluted isocratically with MeOH and seven fractions collected. Weak activity (IC_{50} 18224 ng/mL) was obtained in the fraction K31-2-(3+4)-4 (renamed as K31-a).

The fraction K31-a (97.9 mg) was subjected to further purification using normal phase (Diol, 15 g) chromatography. The column was eluted with a stepped gradient from hexane/EtOAc (1:2) to 100% EtOAc and then to EtOAc/MeOH (1:1). Sixteen fractions were collected and combined after analysis by TLC. After checking with HPLC profiles and ^{1}H NMR spectra, it was found that K31-a-6 to a-11 (5.2 mg) was a pure compound, K31-a-4 was a mixture containing a major compound closely related to K31-a-6 to a-11, K31-a-5 was a mixture of K31-a-4 and K31-a-6 to a-11, K31-a-2 and K31-a-3 were complex mixtures with the major compound being the same to that of K31-a-4, while K31-a-12 to a-16 showed no signals of interest in the ^{1}H NMR experiment.

The further purification of K31-a-4 (9.2 mg) was carried out by preparative HPLC (C_{18} column, reverse phase), eluted with 5% ACN/H_2O isocratically. The expected pure compound was obtained in fraction K31-a-4-2 (1.3 mg).

4.3 Structural Elucidation of K31-a-6 to a-11

K31-a-6 to a-11 was obtained as colourless crystals. The ESMS showed a molecular weight of 154 Daltons [measured 153.00 ($M-H^+$)], indicating a simple structure. The ^1H NMR spectrum (Table 4.2) showed only three aromatic protons. The proton at δ_H 6.81 (d, J = 9.0 Hz) was in the *ortho* position with the proton at δ_H 7.06 (dd, J = 9.0, 3.5 Hz). The later was in the *meta* position with the third proton at δ_H 7.33 (d, J = 3.5 Hz). The APT spectrum (Table 4.2) indicated three tertiary and four quaternary carbons among which six carbons were aromatic. The chemical shifts suggested that the quaternary aromatic carbons at δ_C 149.6 and δ_C 155.7 were oxygen-bearing carbons while the quaternary carbon at δ_C 171.8 was a carbonyl. Considering the molecular weight of 154 Daltons, the molecular formula $C_7H_6O_4$ and the structures as shown in Figure 4.5 were suggested for compound K31-a-6 to a-11.



(a) (b) (c)

Figure 4.5 The suggested structures for K31-a-6 to a-11

To decide the positions of three substituent groups, the chemical shifts of ^1H and ^{13}C NMR of the three suggested structures were estimated by Chem NMR from the H-1 Estimation (in spectra ChemDraw) and ACD/CNMR Predictor. Among the three structures, the estimated chemical shifts of structure (b) were the closest to those of K31-a-6 to a-11. Therefore, compound K31-a-6 to a-11 was proposed as 2,5–dihydroxybenzoic acid. To further confirm this proposal, ^1H and ^{13}C NMR spectra of authentic 2,5–dihydroxybenzoic acid was obtained. Again, both spectra were identical to those of K31-a-6 to a-11 (Table 4.2).

Table 4.2 ^{1}H and ^{13}C NMR data for K31-a-6 to a-11[a]

	K 31-a-6 to a-11		2, 5-dihydroxybenzoic acid	
No.	$^{13}C\ \delta^{c}$	$^{1}H\ \delta^{b}$	$^{13}C\ \delta^{c}$	$^{1}H\ \delta^{b}$
1	112.3 (C)		112.2 (C)	
2	155.7 (C)		155.8 (C)	
3	114.9 (CH)	6.81 (1H, d, 9.0)	114.9 (CH)	6.82 (1H, d, 9.0)
4	124.2 (CH)	7.06 (1H, dd, 9.0, 3.5)	124.3 (CH)	7.07 (1H, dd, 9.0, 3.5)
5	149.6 (C)		149.7 (C)	
6	118.1 (CH)	7.33 (1H, d, 3.5)	118.1 (CH)	7.34 (1H, d, 3.5)
7	171.8 (C)		171.8 (C)	

[a] ^{1}H NMR spectra recorded at 500 MHz in acetone-d_6, ^{13}C NMR spectra recorded at 125 MHz in acetone-d_6.

[b] ^{1}H chemical shift values (δppm from $SiMe_4$) followed by number of protons, multiplicity and coupling constant (J/Hz).

[c] primary, secondary, tertiary and quaternary carbons, assigned by APT.

Biological Activity:

P388 activity: IC_{50} >25000 ng/mL

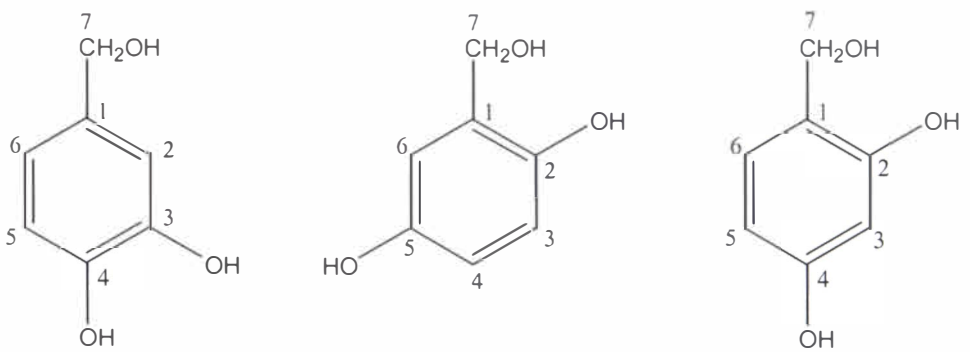
Antimicrobial activity: 2 mm zone of inhibition against *Bacillus subtilis* at a concentration of 80 μg/disk.

Antiviral activity: none detected

Gentisic acid (2,5-dihydroxybenzoic acid) is found in numerous families and genera of green plants[135] and has also been isolated from some species of fungi such as *Penicillium patulum.*[136] Just like many other phenolic acids, gentisic acid also shows antioxidative activity.[137,138]

4.4 Structural Elucidation of K31-a-4-2

K31-a-4-2 was obtained as colourless crystals. The ^{1}H and ^{13}C NMR spectra of K31-a-4-2 (Table 4.3) were similar to those of K31-a-6 to a-11 (Table 4.2) except for the carbonyl carbon at δ_C 171.8 in K31-a-6 to a-11 being replaced by an oxygen-bearing methylene group (δ_C 60.9, δ_H 4.65) in K31-a-4-2. The APT spectrum (Table 4.3) of K31-a-4-2 showed one secondary carbon (δ_C 60.9), three tertiary carbons (δ_C 114.1, 114.3 and 115.7), one quaternary carbon (δ_C 128.5) and two oxygen-bearing quaternary carbons (δ_C 148.1, 150.4). In the ^{1}H NMR spectrum, three aromatic protons and two methylene protons were observed. The aromatic proton at δ_H 6.55 (dd, J = 8.0, 2.5 Hz) was located *ortho* to the proton at δ_H 6.33 (d, J = 8.0 Hz) and *meta* to the proton at δ_H 6.76 (d, J = 2.5 Hz). The chemical shifts of the methylene group (δ_C 60.9, δ_H 4.65) suggested that this is a hydroxymethyl group attached to an aromatic ring. Thus, structures as shown in Figure 4.6 were proposed for K31-a-4-2.



(a) (b) (c)

Figure 4.6 The suggested structures for K31-a-4-2

To determine the positions of three substituent groups, HSQC and CIGAR experiments were carried out. The long-range correlations (Table 4.3, Figure 4.7) of H-7 (δ_H 4.65) to C-1 (δ_C 128.5), C-2 (δ_C 148.1) and C-6 (δ_C 114.3) led to the assignments of C-1, C-2 and C-6, while the long-range correlations of H-3 (δ_H 6.63) to C-5 (δ_C 150.4) and H-4 (δ_H 6.55) to C-6 (δ_C 114.3) led to the assignments of C-3, C-4 and C-5. Thus, the structure as shown in Figure 4.7 was decided for K31-a-4-2.

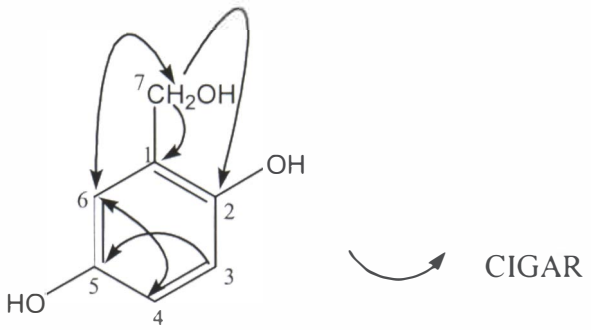


Figure 4.7 Structure for K31-a-4-2

Table 4.3 ^{1}H, ^{13}C, HSQC and CIGAR NMR data for K31-a-4-2[a]

No.	^{13}C δ[c]	^{1}H δ[b]	CIGAR[d]
1	128.5 (C)		
2	148.1 (C)		
3	115.7 (CH)	6.63 (1H, d, 8.0)	128.5, 148.1, 150.4
4	114.1 (CH)	6.55 (1H, dd, 8.0, 2.5)	114.3, 148.1
5	150.4 (C)		
6	114.3 (CH)	6.76 (1H, d, 2.5)	60.9, 114.1, 148.1
7	60.9 (CH_2)	4.65 (2H, s)	114.3, 128.5, 148.1

[a] ^{1}H NMR spectra recorded at 500 MHz in acetone-d_6, ^{13}C NMR spectra recorded at 125 MHz in acetone-d_6.
[b] ^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by number of protons, multiplicity and coupling constant (J/Hz).
[c] primary, secondary, tertiary and quaternary carbons, assigned by APT.
[d] long range ^{1}H-^{13}C correlation from H to C observed in the CIGAR experiment.

Biological Activity:

P388 activity: IC_{50} = 3660 ng/mL

Antimicrobial activity: 3 mm zone of inhibition against *Bacillus subtilis* at a concentration of 40 μg/disk.

Antiviral activity: CYT 4+, TYP 7 for *Herpes simplex virus*, CYT 4+, TYP 7 for

Polio virus at a concentration of 30 μg/disk.

Gentisyl alcohol has been isolated from several species of *Phoma* and *Penicillium*.[139,140] It is reported that gentisyl alcohol, isolated from the culture broth of *Penicillium vulpinum*, exhibited relatively strong cytotoxic activity (IC_{50} 10 μg/mL) against leukaemia cells (L 1210) in tissue culture.[141] Gentisyl alcohol has also been isolated from *Penicillium commune* and shown to be a potent antioxidant and synergist for tocopherol.[142]

4.5 Discussion

Two simple compounds were isolated from the filtrate of a marine *Phoma* sp. (K31) through bioassay-guided separation. The compounds were determined as 2,5–dihydroxybenzoic acid (gentisic acid) and 2,5–dihydroxybenzyl alcohol (gentisyl alcohol) based mainly on ^{1}H, ^{13}C NMR and mass spectra. Although the crude extract of *Phoma* sp. (K31) showed moderate activity (IC_{50} 6248 ng/mL) against the P388 murine leukaemia cell line, this activity was reduced rapidly during chromatography. This was assumed to be due to the active compound(s) being retained on the columns or that the active compound(s) decomposed during chromatography. There was also a possibility that the initial activity was a synergistic action with the less active compounds.

Chapter 5

FUSARIUM SP. (A145)

5.1 Introduction

5.1.1 General

Fusarium species are widely distributed in soils and are known to react very readily to the substrate in which they are growing.[143] This is due to their genetic make up and morphological responses to changes in their environment. Since *Fusarium* strains degenerate very rapidly when cultured on rich media, they should be isolated on poor media and identified as soon as possible after primary isolation.

The most definitive character of this genus is the presence of fusoid macroconidia with a foot cell bearing some kind of heel. This foot cell separates it from *Cylindrocarpon*, the most closely related genus. Some species of *Fusarium* also produce microconidia and terminal or intercalary chlamydospores.[143]

Fusarium species are among the most important plant pathogens, causing a wide range of diseases such as seedling blights, root, stem and ear rots and head blights. They are also responsible for storage rots on tubers and kernels. These diseases can cause not only a serious reduction in grain yield, but also an accumulation of toxic metabolites in plant tissues which contaminate human and animal food.[107]

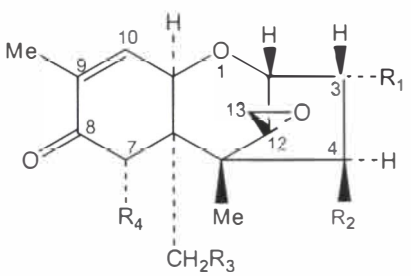
5.1.2 Bioactive metabolites from Fusarium species

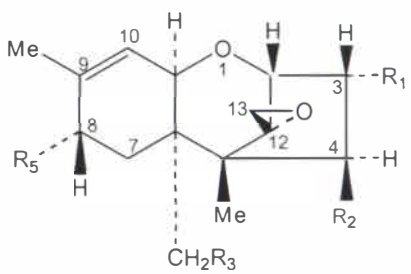
There is an extensive literature on *Fusarium* metabolites, with mycotoxins being the

most important ones reported. This chapter will consider specific groups of metabolites isolated from *Fusarium* spp. based on chemical structures and biological activity.

Trichothecenes

The trichothecenes are a group of mycotoxins which are mainly produced by some species of *Fusarium*. They are chemically the most diverse of all the mycotoxins, with more than 50 trichothecenes having been isolated from this genus.[107] T-2 toxin, HT-2 toxin, nivalenol, diacetoxyscirpenol (DAS) and deoxynivalenol (DON) (Figure 5.1) are the most common compounds of this group. Trichothecenes are classified as sesquiterpenoid derivatives containing a trichothecane ring system with a double bond between C-9, 10 and an epoxy group at C-12, 13 (Figure 5.1). The research on structure-function relationship revealed that a 12-13 epoxide group plays an essential role in the high toxicity of trichothecenes.[144,145] When the epoxide group is modified the toxicity of the compound is lost.





	R_1	R_2	R_3	R_4
Nivalenol	OH	OH	OH	OH
DON	OH	H	OH	OH

	R_1	R_2	R_3	R_5
T-2 toxin	OH	OAc	OAc	$OCOCH_2CH(CH_3)_2$
HT-2 toxin	OH	OH	OAc	$OCOCH_2CH(CH_3)_2$
DAS	OH	OAc	OAc	H

Figure 5.1 Structures of some trichothecenes

Trichothecenes have been implicated in human mycotoxicoses such as Alimentary Toxic Aleukia (ATA) in Russia.[146] The occurrence of trichothecenes in foods and feeds is often associated with intoxications in humans and animals and is responsible for gastrointestinal disorders,[147] skin inflammation,[148] blood disorders, destruction of bone marrow and nerve disorders.[149]

The correlation between trichothecene production and *Fusarium* pathogenicity in cereals has long been suggested.[150] Some trichothecenes have proved to be a major component for fusariosis of wheat heads.[151]

Although known as mycotoxins, trichothecenes also exhibit other activities such as high antimalarial activity[152] and antitumour properties.[153]

Zearalenones

Several species of *Fusarium* are capable of producing zearalenones.[154,155] Zearalenone (Figure 5.2) and its derivatives are oestrogen mimetics and consist of a resorcinol moiety fused to a 14-member macrocyclic lactone. The natural occurrence of zearalenone has been detected in a number of feeds and grains. As a result zearalenones have been implicated in numerous incidences of reproductive disorder (e.g. hyperoestrogenism; vulvovaginitis; infertility; reduced litter size and weak offspring) particularly in swine, and to a lesser extent, in poultry and laboratory rats and mice.[156,157]

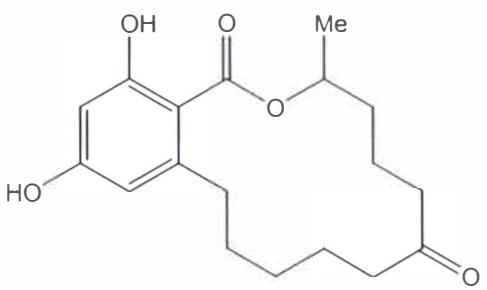
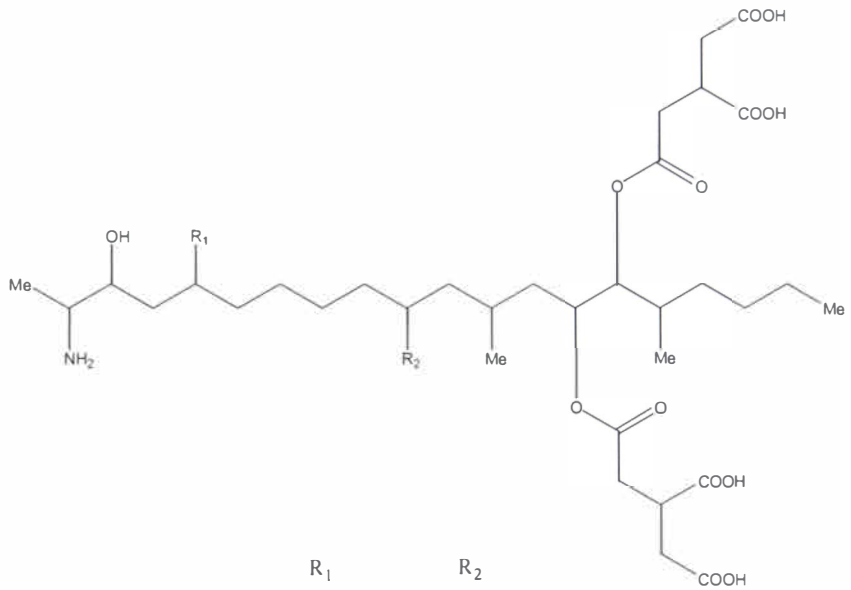


Figure 5.2 Structure of zearalenone

Fumonisins

Fumonisins are another important class of mycotoxins produced by *Fusarium moniliforme* and other *Fusarium* species grown on cereal and cereal-based food products.[158,159] They are sphingosine analogues characterized by a 19- or 20-carbon aminopolyhydroxyalkyl chain which is diesterified with propane-1,2,3-tricarboxylic acid. Fumonisins are further classified as A-, B-, C-, and P-series, with fumonisins B_1, B_2 and B_3 being the major ones produced in nature (Figure 5.3).[160-162]



	R_1	R_2
Fumonisin B_1	OH	OH
Fumonisin B_2	OH	H
Fumonisin B_3	H	OH

Figure 5.3 Structures of fumonisins (B-series)

Fumonisins have been pointed to as a natural cause of leukoencephalomalacia in horses[163] and pulmonary edema in pigs.[164] Evidence also suggests that consumption of fumonisin-contaminated corn contributes to human oesophageal cancer in Southern Africa and China[165,166] and is a risk factor for promotion of human primary liver cancer.[167]

Fumonisin B_1 (FB_1), the most important compound of the group, has been reported to be a hepatotoxic and hepatocarcinogenic agent in rats.[168] FB_1 has also been shown to inhibit sphingolipid biosynthesis, which has been correlated with both animal toxicity and carcinogenicity.[169,170]

Pigments:

More than 30 pigments have been isolated from *Fusarium* species. They are naphazarin derivatives such as aurofusarin (yellow prisms) from *F. culmorum,*[171] bostrycoidin (red plates) from *F. solani,*[172] bikaverin (red crystals) from *F. oxysporum,*[173] fusarubin (red prisms) and marticin (dark red needles) from *F. martii var. piso*., *F. solani*, *F. solani var. minus* and *F. javanicum*[174] and solanilo (dark red

needles) from *F. solani*.[175] Many of the pigments isolated from *Fusarium* species are reported to have antibiotic, antitumour, insecticidal and phytotoxic properties.[176]

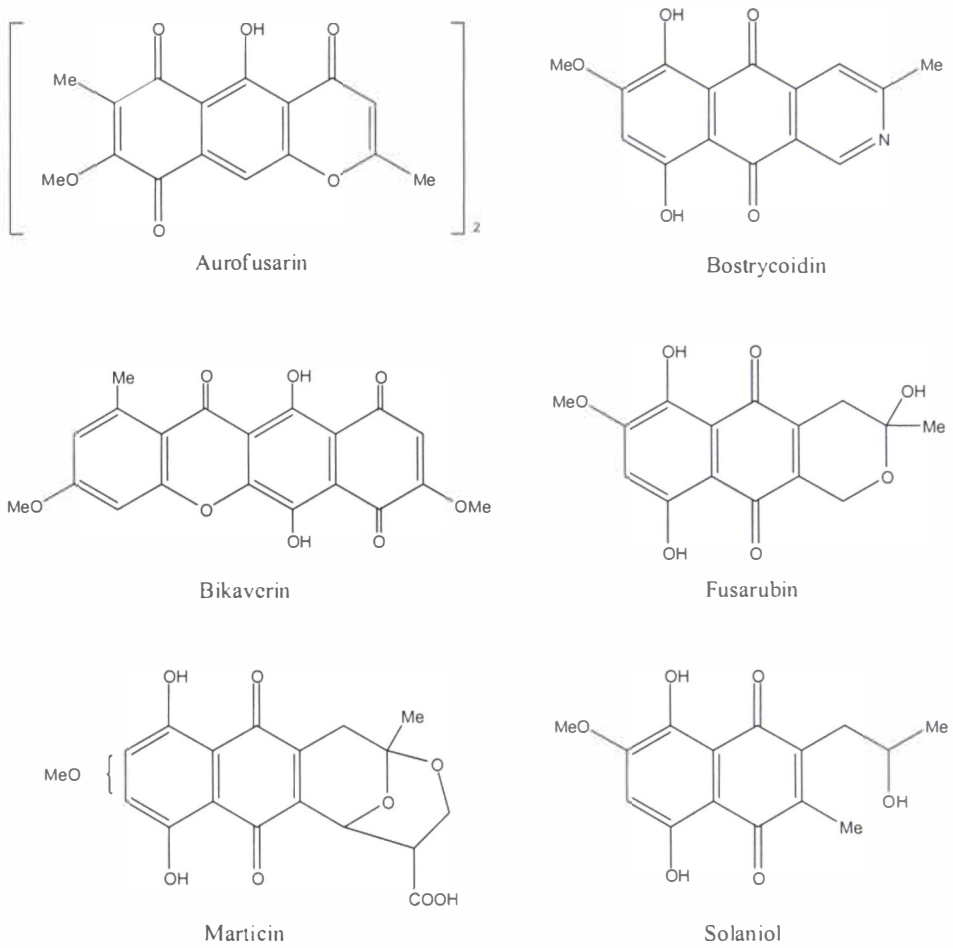


Figure 5.4 Structures of pigments isolated from *Fusarium* species

5.2 Culturing and Isolation

5.2.1 Culturing and extraction

A fungal strain CANU A145, isolated from saline lake sand in western Australia, was identified as belonging to the genus *Fusarium* (Figure 5.5). The isolate was grown on freshly prepared PDA Petri dishes for 7 days at 25 °C. Then mycelial disks (8 mm in diameter) were cut from the agar and transferred into 4 litres of half-strength potato dextrose broth. The broth was incubated for 5 weeks at 26 °C under agitating

conditions. The culture was filtered under suction and the mycelium and filtrate extracted separately with EtOAc. The EtOAc extracts were then evaporated under reduced pressure to give 243.6 mg of filtrate extract and 674.8 mg of mycelial extract. On the P388 assay, the filtrate extract showed significant activity (IC_{50} < 975 ng/mL) while the mycelial extract exhibited very weak activity (IC_{50} 23167 ng/mL). The filtrate extract was therefore selected for further purification.

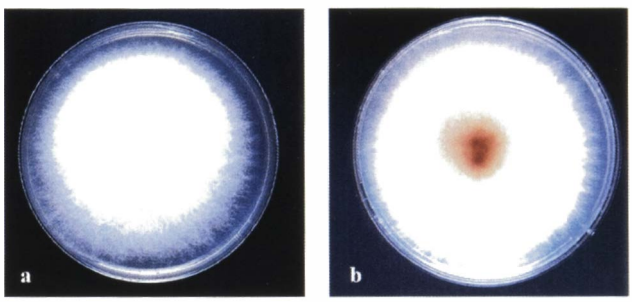
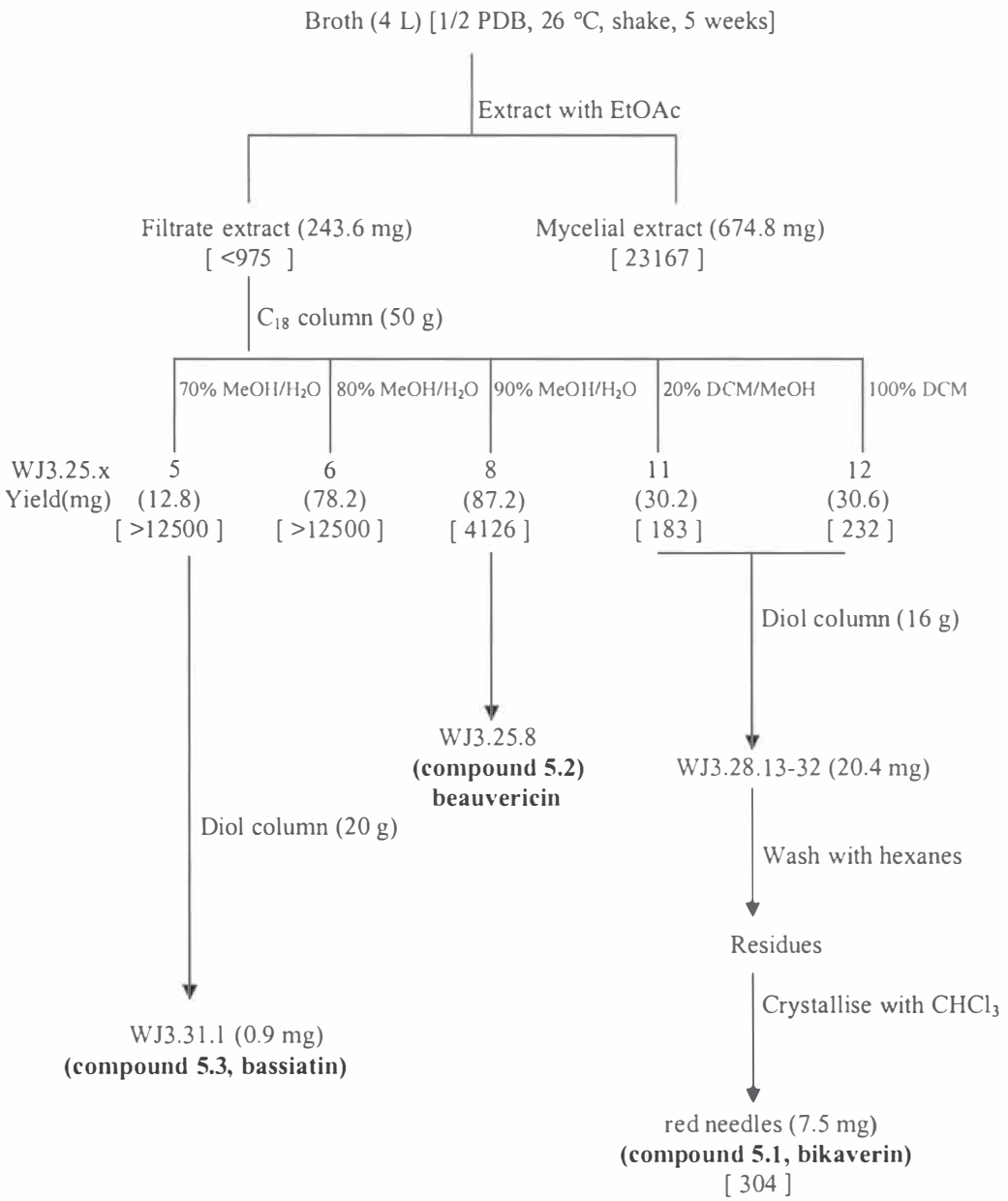


Figure 5.5 Colony of *Fusarium* sp. (A145) on PDA (7 days, 25 °C)
(a) top; (b) reverse

5.2.2 Isolation of active metabolites

An outline of the isolation of bioactive compounds from *Fusarium* sp. (A145) is illustrated in **Scheme 5.1**.

Scheme 5.1 Isolation of Active Compounds from *Fusarium* sp. (A145)



[ ]: P388 activity (IC_{50} = ng/mL)

The filtrate extract (243.6 mg) was applied to a C_{18} (reverse-phase) column, eluted with a stepped gradient from 10% MeOH/H_2O to MeOH and then CH_2Cl_2. Fourteen fractions were collected. Fractions WJ3.25.7 to WJ3.25.12 were active in the assay against the P388 cell line. By checking with HPLC traces (ACN/H_2O gradient, C_{18}

column, standard conditions) and ^{1}H NMR spectra of the active fractions, fraction WJ3.25.8 (eluted with 90% MeOH/H_2O) afforded a pure compound (87.2 mg, compound **5.2**). WJ3.25.11 and 12 (eluted with 20% DCM/MeOH and 100% DCM respectively) were combined for further purification. WJ3.25.9 and 10 were found to be a mixture of WJ3.25.8 and WJ3.25.11+12, and no further work was required for these two fractions. WJ3.25.7 was a mixture containing more than 90% of WJ3.25.8. Since there was only 6.6 mg of this fraction, no further purification was carried out.

Fraction WJ3.25.11+12 (60.8 mg) was chromatographed on a Diol column (normal phase, 16 g) using a gradient solvent system from hexanes to DCM then to MeOH as eluents. Thirty seven subfractions were collected. Subfractions WJ3.28.13 to 32, eluted with 70% - 90% DCM/hexanes, 100% DCM and 50% MeOH/DCM, contained compound **5.1**. Because of its poor solubility, compound **5.1** was always mixed with fatty acid in the subfractions collected. Subfractions WJ3.28.13 to 32 were combined and dried, and then washed three times with hexanes to remove the fatty acid. The residue was crystallised with chloroform to give red needles (7.5 mg).

Although fraction WJ3.25.5 was inactive in the P388 assay, its ^{1}H NMR spectrum indicated that it might contain a metabolite closely related to compound **5.2**. Therefore, this fraction was purified through a Diol column, eluted with a gradient solvent system consisting of hexanes - DCM - MeOH. Twenty one subfractions were collected. Subfraction WJ3.31.1, eluted with 100% hexanes to 60% DCM/hexanes, afforded pure compound **5.3** (0.9 mg).

5.3 Structural Elucidation of Compound 5.1 (Bikaverin)

Compound **5.1** was obtained as red needles. The HREIMS showed a peak at 382.06961 (M^+) indicating the formula $C_{20}H_{14}O_8$ (calcd: 382.06887, M^+). The ^{1}H NMR spectrum (Table 5.1) showed signals of two methoxy groups (δ_H 3.93, 3.96), one methyl group (δ_H 2.86), and three aromatic-type protons (δ_H 6.35, 6.80, 6.93).

Assignment of peaks was complicated by the absence of hydrogen, and by the poor solubility of compound **5.1** in most solvents. No ^{13}C NMR spectrum was achieved and very little information could be obtained from the CIGAR spectrum (Table 5.1)

due to its low solubility in the ordinary solvents for NMR spectroscopy.

From its physical characteristics (Table 5.2), ^{1}H NMR spectrum and molecular formula, a partial structure with an aromatic ring and a quinonoid was proposed for compound **5.1**. After a literature search for pigments isolated from *Fusarium* species, one compound, bikaverin, seemed promising. Comparison of ^{1}H NMR (Table 5.1) and mass spectrum with those of the literature proved that compound **5.1** was identical with bikaverin (Figure 5.6), previously confirmed by a series of chemical reactions and single-crystal X-ray diffraction.[173,177,178]

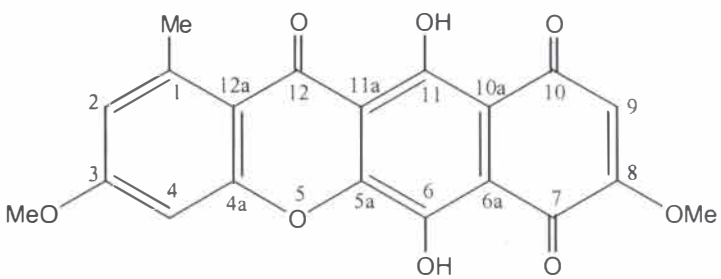


Figure 5.6 Structure of bikaverin

Table 5.1 ^{1}H, ^{13}C, HSQC, COSY and CIGAR NMR data for compound **5.1**[a]

no.	$^{13}C\ \delta$[c]	$^{1}H\ \delta$[b]	COSY	CIGAR[d]	$^{1}H\ \delta$[e]
1- Me	23.5	2.86 (3H, s)	6.80 (w)	144.3, 117.5	2.86
2	118.0	6.80 (1H, d, 1.0)	2.86 (w), 6.93		6.77
3-OMe	57.0	3.96 (3H, s)		159.0	3.95
4	90.9	6.93 (1H, d, 1.5)	6.80		6.92
8-OMe	56.0	3.93 (3H, s)		164.5	3.93
9	112.5	6.35 (1H, s)			6.35

[a] ^{1}H NMR spectra recorded at 500 MHz in $CDCl_3$, ^{13}C NMR spectra recorded at 125 MHz in $CDCl_3$.
[b] ^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by number of protons, multiplicity and coupling constant (J/Hz)
[c] ^{13}C chemical shift values were measured by HSQC and CIGAR spectra.
[d] long range ^{1}H-^{13}C correlation from H to C observed in the CIGAR experiment.
[e] ^{1}H chemical shifts on the literature report (in $CDCl_3$)[177].

Table 5.2 UV, IR and MS data for compound 5.1

UV ($CHCl_3$): λ_{max} ($CHCl_3$) nm (log ε) 254 (4.18), 278 (4.17) and 520 (3.70)

IR (KBr): ν_{max} 1672, 1654, 1616, 1591 and 1569 cm^{-1}

MS: *m/e* 382 (M^+, $C_{20}H_{14}O_8$), 367 (M^+ - CH_3, $C_{19}H_{11}O_8$), 353 (M^+ - CHO, $C_{19}H_{13}O_7$), 339 (367 – CO, $C_{18}H_{11}O_7$), 311(339 – CO, $C_{17}H_{11}O_6$)

Biological Activity:

P388 activity: IC_{50} = 304 ng/mL

Antimicrobial activity: 2 mm zone of inhibition against *Trichophyton mentagrophtes* at a concentration of 60 μg/disk.

Antiviral activity: none detected

Bikaverin is a red pigment produced by many fungal isolates and commonly occurs in *Fusarium* species.[179] It has been shown that bikaverin is identical with 'lycopersin' from *Fusarium lycopersici*[173,180,181] and with 'mycogonin' from *Mycogone jaapii*.[182] Bikaverin has antitumour activity inhibiting Erlich ascites carcinoma, leukaemia L5178, and sarcoma 37 with ED_{50} values of 0.5, 1.4 and 4.2 μg/mL, respectively.[183] Bikaverin also shows marked and specific antiprotozoal activity. It causes cessation of extension growth and induces vacuolation and other morphological symptoms of ageing when applied to a variety of fungi.[173,184] Bikaverin has also been found to inhibit mitochondrial functions in isolated rat liver and to be an efficient haemolytic agent.[185]

5.4 Structural Elucidation of WJ3.31.1 (Bassiatin)

WJ3.31.1 was obtained as colourless powder. The HREIMS showed molecular weight at 261.13685 (M^+), indicating a molecular formula $C_{15}H_{19}NO_3$ (calculated mass: 261.13649, M^+). The ^1H NMR, APT and HSQC spectra of WJ3.31.1 (Table 5.3) indicated the presence of two aliphatic methyl groups (δ_H 0.76 and 0.85), one *N*-methyl group (δ_C 32.6, δ_H 3.02), one methylene group (δ_H 3.20 and 3.28), three

methine groups (δ_H 2.30, 2.99 and 4.40), one aromatic quaternary (δ_C 134.3) and two carbonyl carbon atoms (δ_C 165.7 and 167.5). The IR absorptions at 1745 and 1670 cm^{-1} indicated that these two carbonyl carbons were an ester carbonyl and an amide carbonyl respectively. Although fifteen carbons were suggested by HREIMS, only thirteen carbons could be observed from APT and CIGAR spectra. This indicated there were two carbons with identical chemical shifts with others. These two carbons should be aromatic carbons because five aromatic protons (δ_H 7.13 – 7.33) were measured by integration of ^{1}H NMR data while only four aromatic carbon signals (δ_C 134.3, 128.4, 129.4 and 129.9) can be seen on the ^{13}C NMR spectrum. Thus, a monosubstituted phenyl group was proposed in the structure. The methine group at δ_C 81.4 (δ_H 2.99) was ascribed to be an oxygen-bearing methine by its chemical shift.

By examining the COSY spectrum (Table 5.3), a partial structure $-CH_2$-CH was suggested by the correlations of methylene protons (δ_H 3.20, 3.28) to a methine proton at δ_H 4.40. The partial structure $(CH_3)_2$ CH-CH-O- was deduced by the correlations of H-7 (δ_H 2.30) to H-8 (δ_H 0.85), H-7 to H-9 (δ_H 0.76), and H-7 to H-6 (δ_H 2.99).

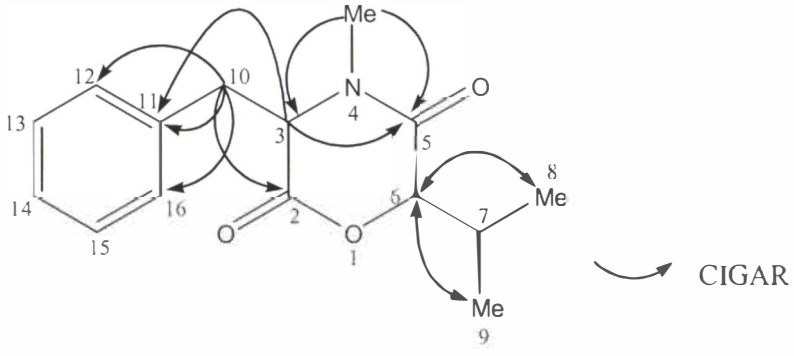


Figure 5.7 Structure of WJ3.31.1 (bassiatin)

The connection of these functional groups was achieved mainly by analysis of the CIGAR spectrum (Table 5.3). The long range correlations of the proton at δ_H 3.20 in the methylene group to the aromatic carbons at δ_C 129.9 (C-12, C-16) and δ_C 134.3 (C-11) and δ_H 4.40 (H-3) to δ_C 134.3 (C-11), led to the connection of C-10 to the phenyl group (Figure 5.7). The long range correlations of δ_H 3.02 (N-CH_3) to δ_C 62.9 (C-3) and δ_C 165.7 (C-5), and δ_H 4.40 (H-3) to δ_C 165.7 (C-5) resulted in the connection of C-3 to N-4 then to C-5. The connection between C-2 and C-3 was

assumed by the long rang correlation of δ_H 3.20 (H-10) to δ_C 167.5 (C-2). Finally, the planar structure as shown in Figure 5.7 was suggested for WJ3.31.1. From a literature search, this compound was found to be bassiatin, previously isolated from *Beauveria bassiana*.[186]

The stereochemistry of WJ3.31.1 was determined as shown in Figure 5.8 by comparing the ^1H and ^{13}C NMR data (Table 5.3) and the optical rotation ($[\alpha]_D^{20}$ +120.0 °, c 0.2, $CHCl_3$) of WJ3.31.1 with the four isomers synthesized by Kagamizono *et al.*[186]

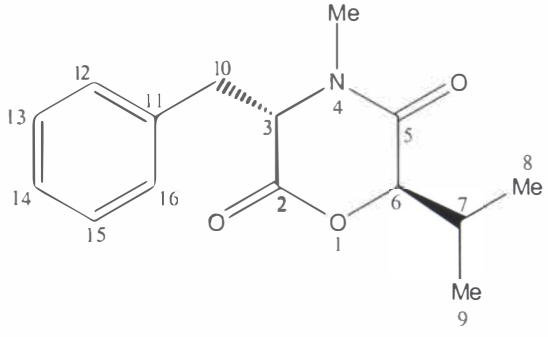


Figure 5.8 Stereostructure of WJ3.31.1 (bassiatin)

Biological Activity:

P388 activity: IC_{50} >12500 ng/mL

Antimicrobial activity: none detected

Antiviral activity: none detected

Bassiatin was isolated as a platelet aggregation inhibitor. The inhibitory activity (IC_{50}) of bassiatin against rabbit platelet aggregation induced by ADP, collagen and arachidonic acid was 1.9 x 10^{-4} M, 3.8 x 10^{-4} M and 3.8 x 10^{-4} M, respectively, only slightly less active than adenosine.[186]

Table 5.3 ^{1}H, ^{13}C, HSQC, COSY and CIGAR NMR data for WJ3.31.1[a]

no.	^{13}C δ [c]	^{1}H δ [b]	COSY	CIGAR[d]	
				H ⟶ C	C ⟶ H
2	167.5 (C)				3.20
3	62.9 (CH)	4.40 (1H, t, 4.5)	3.20, 3.28	134.3(w), 165.7	3.02, 3.20(w)
5	165.7 (C)				3.02, 4.40(w)
6	81.4 (CH)	2.99 (1H, s)	2 30	15.3, 18.8(w), 29 8(w)	0.76, 0.85
7	29.8 (CH)	2.30 (1H, m)	0.76, 0.85, 2.99		0.76, 0.85, 2.99
8	18.8 (CH_3)	0.85 (3H, d, 7.0)	2.30	15.3, 29.8, 81.4	0.76, 2.99
9	15.3 (CH_3)	0.76 (3H, d, 6.5)	2.30	18.8, 29.8, 81.4	0.85
10	37.3 (CH_2)	3.20 (1H, dd, 14.0, 5.0)	3.28, 4.40	62.9(w), 129.9, 134.3, 167.5	
		3.28 (1H, dd, 14.0, 4.5)	3.20, 4.40	129.9	
11	134.3 (C)				3.20, 4.40, 7.33
12, 16	129.9 (CH)	7.13 (2H, m)	7.33	128.4	3.20, 3.28
13, 15	128.4 (CH)	7.33 (2H, m)	7.13	134.3	7.13
14	129.4 (CH)	7.32 (1H, m)	7.33		
N-CH_3	32.6 (CH_3)	3.02 (3H, s)		62.9, 165.7	

[a] ^{1}H NMR spectra recorded at 500 MHz in $CDCl_3$, ^{13}C NMR spectra recorded at 125 MHz in $CDCl_3$.
[b] ^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by number of protons, multiplicity and coupling constant (J/Hz).
[c] primary, secondary, tertiary and quaternary carbons, assigned by APT.
[d] long range ^{1}H-^{13}C correlation from H to C or C to H observed in the CIGAR experiment.

5.5 Structural Elucidation of WJ3.25.8 (Beauvericin)

WJ3.25.8 was obtained as pale yellow needles. The ^{1}H and ^{13}C NMR spectra (Table 5.4) of WJ3.25.8 showed similarities to that of WJ3.31.1 (Table 5.3) in terms of the observed chemical shifts and the multiplicity of the peaks: two methyl doublets (δ_H 0.40 and 0.79), one *N*-methyl singlet (δ_H 3.00), one methylene group (δ_H 2.96 and 3.36, AB system), three methine groups (δ_H 1.99, multiplet, δ_H 4.88, doublet and δ_H 5.52, quartet), five aromatic protons (δ_H 7.18 – 7.24), one aromatic quaternary (δ_C

136.8) and two carbonyl carbon atoms (δ_C 169.6 and 170.1). The presence of a phenyl group was suggested by the NMR data of C-11 to C-16 (Table 5.4). The methine signal at δ_H 4.88 (δ_C 75.6) were assumed to be an oxygen-bearing methine group by its chemical shift.

In the COSY spectrum (Table 5.4) of WJ3.25.8, couplings were observed between two methyl groups (δ_H 0.40 and 0.79) and one methine group at δ_H 1.99, which in turn was coupled with another methine group at δ_H 4.88. This led to the partial structure $(CH_3)_2$ CH-CH-O-. The fragment -CH_2-CH- was determined by the couplings of the methylene group (δ_H 2.96 and 3.36) with the third methine group (δ_H 5.52).

In the CIGAR spectrum (Table 5.4), long-range correlations were observed between *N*-methyl protons (δ_H 3.00) to a carbonyl carbon at δ_C 169.6 (C-5) and to a methine carbon at δ_C 57.4 (C-3). This led to the connection of C-3 to N-4 then to C-5. The long-range correlations of the methylene (δ_H 2.96 and 3.36) protons and the oxygen-bearing methine proton (δ_H 4.88) to another carbonyl carbon (δ_C 170.1) resulted in the connection of C-10 to C-2 through C-3 and C-2 to C-6 through an oxygen respectively. The correlation of the proton at δ_H 1.99 (H-7) to the carbonyl carbon at δ_C 169.6 (C-5) led to the connection of C-5 to C-6. Finally, the connection of C-10 to the phenyl group was determined by long-range correlations observed between the methylene group (H-10a, H-10b) to the quaternary carbon at δ_C 136.8 (C-11) and the aromatic carbon at δ_C 129.0 (C-12, C-16). From these results, the planar structure of WJ3.25.8 was proposed as depicted in Figure 5.9, which is the same as that of WJ3.31.1 (Figure 5.7).

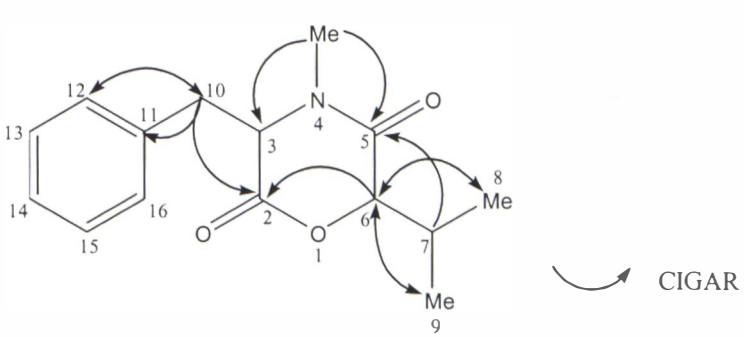


Figure 5.9 Structure I for WJ3.25.8

At this point, WJ3.25.8 was assumed to be an isomer of WJ3.31.1, but mass spectra indicated the molecular weight of WJ3.25.8 was 783 Daltons [EI: 783 (M^+), ES: 784.2386 $(M + H)^+$, 782.2600 $(M - H)^+$] rather than 261 Daltons as found for WJ3.31.1. Thus, WJ3.25.8 was proposed to be a trimer of WJ3.31.1 and this was supported by the high resolution mass spectrum (HREIMS), which suggested the molecular formula $C_{45}H_{57}N_3O_9$ (M^+ 783.41100, calculated mass: 783.40948). The symmetry and cyclic nature of WJ3.25.8 was indicated by the simplicity of the ^1H and ^{13}C NMR spectra (Figure 5.11, Figure 5.12), thus, the planar structure II, a cyclodepsipeptide, was suggested for WJ3.25.8 (Figure 5.10). This structure is identical to beauvericin, an antibiotic of the enniatin family. The ^1H and ^{13}C NMR data, mass spectrum and optical rotation ($[\alpha]_D^{20}$ +61.0 °, c=1, methanol) of WJ3.25.8 were in good agreement with those reported for beauvericin.[187,188] The absolute stereochemistry has been established as a cyclic hexadepsipeptide of alternating L-*N*-methylalanyl and D-α-hydroxyisovaleryl residues by Hamill *et al.* in 1969.[187]

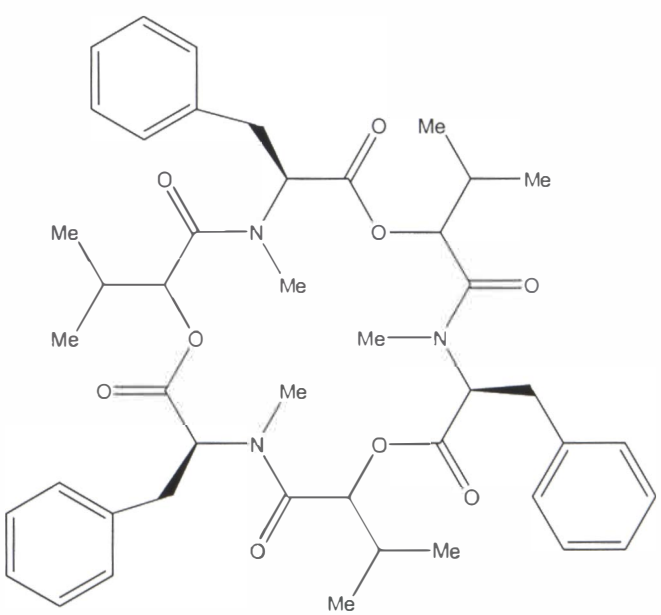


Figure 5.10 Structure II for WJ3.25.8

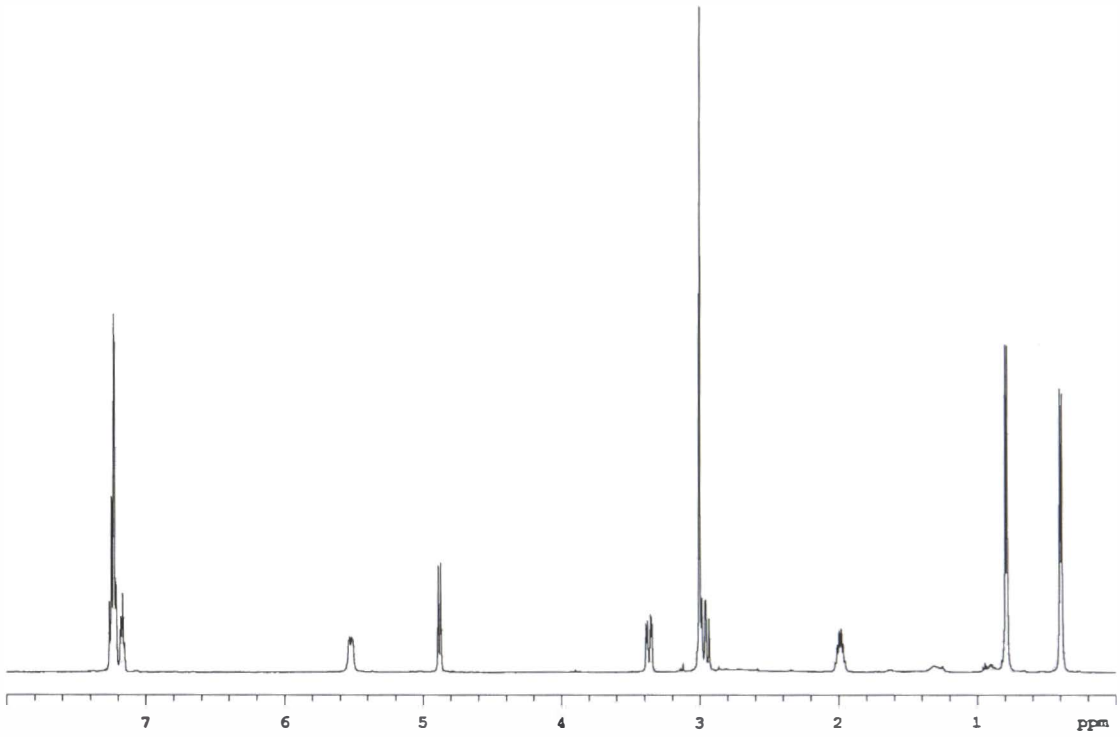


Figure 5.11 ^{1}H NMR spectrum of WJ3.25.8 (in $CDCl_3$)

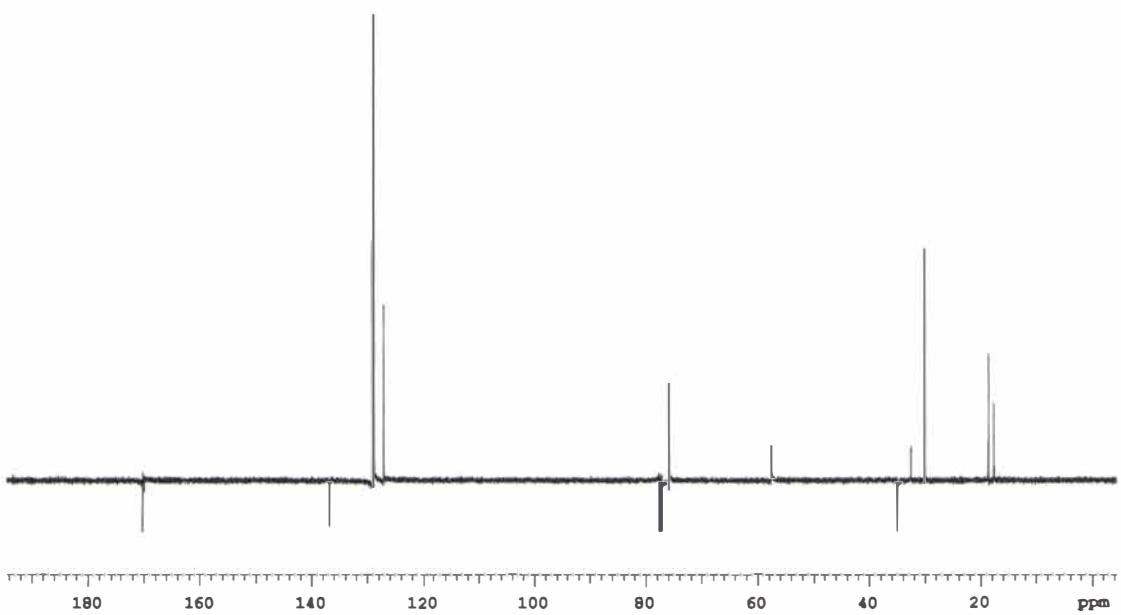


Figure 5.12 ^{13}C NMR (APT) spectrum of WJ3.25.8 (in $CDCl_3$)

Table 5.4 ^{1}H, ^{13}C, HSQC, COSY and CIGAR NMR data for WJ3.25.8[a]

no.	^{13}C δ [c]	^{1}H δ [b]	COSY	CIGAR[d]	
				H ⟶ C	C ⟶ H
2	170.1 (C)				2.96, 3.36, 4.88
3	57.4 (CH)	5.52 (1H, dd, 11.0, 4.0)	2.96, 3.36		3.00, 3.36, 2.96
5	169.6 (C)				1.99, 3.00
6	75.6 (CH)	4.88 (1H, d, 8.5)	1.99	17.6, 18.5, 29.9, 170.1	1.99, 0.40, 0.79
7	29.9 (CH)	1.99 (1H, m)	0.40, 0.79, 4.88	17.6, 18.5, 75.6, 169.6	0.40, 0.79, 4.88
8	18.5 (CH_3)	0.79 (3H, d, 6.5)	1.99	17.6, 29.9, 75.6	0.40, 1.99, 4.88
9	17.6 (CH_3)	0.40 (3H, d, 7.0)	1.99	18.5, 29.9, 75.6	0.79, 1.99, 4.88
10	34.9 (CH_2)	H10a: 3.36 (1H, dd, 14.5, 5.0)	2.96, 5.52	57.4, 136.8, 129.0, 170.1	7.23
		H10b: 2.96 (1H, m)	3.36, 5.52	57.4, 136.8, 129.0, 170.1	7.23
11	136.8 (C)				2.96, 3.36, 7.24
12, 16	129.0 (CH)	7.23 (2H, m)		34.9, 127.0, 128.7	7.18, 7.24
13, 15	128.7 (CH)	7.24 (2H, m)	7.18	129.0, 136.8	7.23
14	127.0 (CH)	7.18 (1H, m)	7.24	129.0	7.23
N-CH_3	32.4 (CH_3)	3.00 (3H, s)		169.6, 57.4	

[a] ^{1}H NMR spectra recorded at 500 MHz in $CDCl_3$, ^{13}C NMR spectra recorded at 125 MHz in $CDCl_3$.
[b] ^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by number of protons, multiplicity and coupling constant (J/Hz).
[c] primary, secondary, tertiary and quaternary carbons, assigned by APT.
[d] long range ^{1}H-^{13}C correlation from H to C or C to H observed in the CIGAR experiment.

Biological Activity:

P388 activity: IC_{50} = 4126 ng/mL

Antimicrobial activity: 1 mm, 3 mm and 2 mm zones of inhibition against *Bacillus subtilis*, *Candida albicans* and *Trichophyton mentagrophtes* respectively at a concentration of 40 μg/disk.

Antiviral activity: none detected

Beauvericin was first isolated as a substance toxic to brine shrimp (*Artemia salina*) from *Beauveria bassiana.*[187] It was subsequently isolated from other fungi such as *Fusarium semitectum* and *F. moniliforme var. subglutinans* as a toxic principle against Colorado potato beetles,[189] from powdered Bai Jiang Yong (a dried, *Beauveria bassiana*-fermented Bomlyx mari pupa, used as an anticonvulsant in traditional Chinese medicine,[190] and from the entomopathogenic fungi *Paecilomyces fumoso-roseus*[191] and *P. tenuipes.*[192] Beauvericin showed insecticidal activity against mosquito larvae.[187,188] It also showed an inhibitory effect on a high potassium-induced tonic contraction in guinea pig tenia coli. The mechanism may be due to the noncompetitive inhibition of Ca^{2+} entry through the voltage-dependent Ca^{2+} channel of the intestinal smooth muscle cell. It is also reported that beauvericin inhibited ADP induced platelet aggregation with an IC_{50} of 6.4×10^{-5} M.[186] Synthetic beauvericin had strong antimicrobial activity *in vitro* against a variety of microorganisms, including *Candida albicans*, *Escherichia coli*, *Sarcina lutea* and *Staphylococcus aureus.*[193]

5.6 Discussion

Three compounds were isolated from the filtrate of a marine *Fusarium* sp. (A145) through bioassay-guided separation. The compounds were structurally assigned as bikaverin (compound 5.1), bassiatin (WJ3.31.1) and beauvericin (WJ3.25.8) based mainly on MS, ^{1}H and ^{13}C NMR and a series of 2D NMR spectra. Bikaverin showed strong activity (IC_{50} 304 ng/mL) while beauvericin exhibited moderate activity (IC_{50} 4126 ng/mL) in the assay against the P388 murine leukaemia cell line. The isolation of bassiatin is the first report of its production by a *Fusarium* species. Bassiatin consists of *N*-methyl-*L*-phenylalanine and (-)-(*R*)-2-hydroxy-3-methyl butyric acid and beauvericin is a cyclic trimer of bassiatin. In the study by Kagamizono and co-

workers,[186] both bassiatin and beauvericin showed inhibitory activities against platelet aggregation. Although a series of activities has been reported for beauvericin, the literature on the bioactivity of bassiatin is very limited. In light of the structural similarities between these two compounds, it may be useful to investigate other activities of bassiatin, especially insecticidal activity.

Chapter 6

ASPERGILLUS SP. (A151)

6.1 Introduction

6.1.1 General

The genus *Aspergillus* has a worldwide distribution and is one of the most common of all groups of fungi. Species of *Aspergillus* are among the most economically important fungi, being very widely used for synthesis of chemicals, biosynthetic transformations and enzyme production. Since ancient times, *Aspergillus* species have been used in the preparation of soy sauce in China and other Asian countries. The ability of these fungi to produce several organic acids, especially citric acid, has created major industries worldwide. *Aspergillus* enzymes are used for such diverse applications as starch processing, cheese manufacturing, juice clarification, brewing, dough conditioning, wine modification, food preservation and instant tea production. They are, however, also one of the greatest contaminants of natural and man-made organic products, and a few species are pathogenic to man and animals. The aspergilli are also one of the most important mycotoxin-producing groups of fungi when growing as contaminants on cereals, oil seeds and other foods.

6.1.2 Identification of Aspergillus species

The genus *Aspergillus* now contains more than 200 species[194] and is a relatively large taxon among the Hyphomycetes. It is characterised by the formation of conidiophores with large, heavy walled stipes and swollen apices, termed vesicles. Vesicles bear

crowded phialides or metulae and phialides, which characteristically are all borne simultaneously. This character unequivocally distinguishes *Aspergillus* from *Penicillium*. Differentiation of *Aspergillus* species is based primarily on head complexity- whether metulae and phialides are produced, or phialides alone and conidial colour. In species lacking teleomorphs, *Aspergillus* colony coloration is dominated by conidial colour. No other Hyphomycete genus produces conidia of such diverse colours, which are consistently associated with particular species.[195]

6.1.3 Bioactive metabolites from Aspergillus species

Aspergilli readily produce a variety of secondary metabolites when grown on artificial media in the laboratory and some of these occur naturally when grown on food or feed products. Literature on secondary metabolites isolated from the *Aspergillus* genus is vast: more than 900 compounds have been recorded in Antibase,[75] many of which have overt biological activity including mammalian toxicity. A number of metabolites are important in human health and include:

Aflatoxins

Aflatoxins, produced mainly by *Aspergillus flavus*, *A. parasiticus* and *A. nominus*, are by far the most widely studied mycotoxins and poisoned thousands of poultry, pigs and trout in the early 1960s. They are highly substituted coumarins containing a double furan ring. The four main naturally produced aflatoxins are B_1, B_2, G_1 and G_2 (Figure 6.1).

Aflatoxin B_1 is hepatotoxic and one of the most potent animal carcinogens known. In human liver cell tissue culture, doses of 10 μg/mL are lethal.[196] Since aflatoxins were associated with food contamination, the content of aflatoxins in foods and feeds was eventually regulated in many countries.[197] The possibility that aflatoxins are responsible for the high incidence of liver cancer in parts of Africa where people consume groundnut products was suggested as early as 1962.[198] Yet today, in populations with relatively high exposure, a role for aflatoxins as a risk factor for primary liver cancer in humans is still not clear.[199]

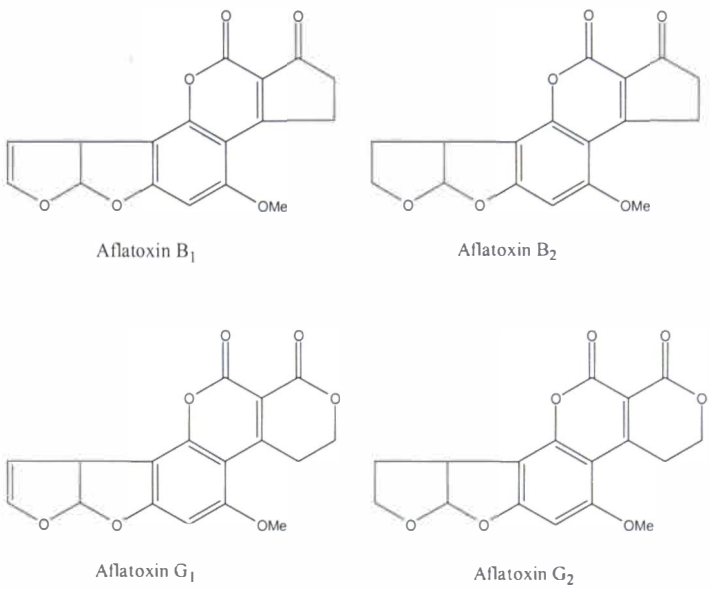
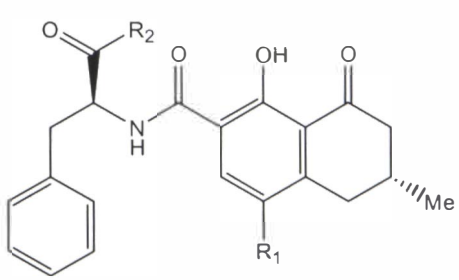


Figure 6.1 Structures of aflatoxins

Ochratoxins

Ochratoxins are isocoumarin compounds (Figure 6.2) produced by *A. ochraceus* and related species.[200] Ochratoxin A (OTA) is a potent nephrotoxin, teratogen, and carcinogen[201] while its natural analogs, such as ochratoxin B and C, showed no or much less toxicity in animals than OTA.[202,203] Inhalation of OTA can lead to renal failure[204] and evidence points strongly to OTA being the cause of Balkan endemic nephropathy.[205,206]



	R_1	R_2
Ochratoxin A	Cl	OH
Ochratoxin B	H	OH
Ochratoxin C	Cl	OCH_2CH_3

Figure 6.2 Structures of ochratoxins

Sterigmatocystins

Sterigmatocystin (Figure 6.3) and its derivatives such as dihydrosterigmatocystin, O-methylsterigmatocystin and O-methyldihydrosterigmatocystin are produced mainly by

A. versicolor.[207] Sterigmatocystin is an intermediate in the biosynthetic pathway leading to the aflatoxins. While acutely toxic and carcinogenic[208] it is not as toxic as aflatoxin B_1. It is also reported that sterigmatocystin and 5-methoxysterigmatocystin caused significant inhibition of transplanted mouse leukaemia P-388 and L-1210 cells.[209]

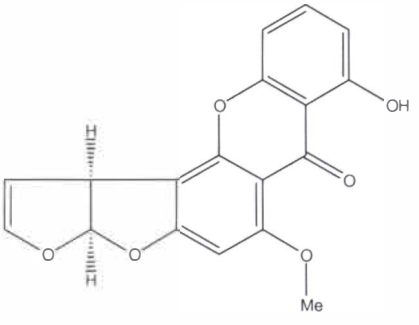
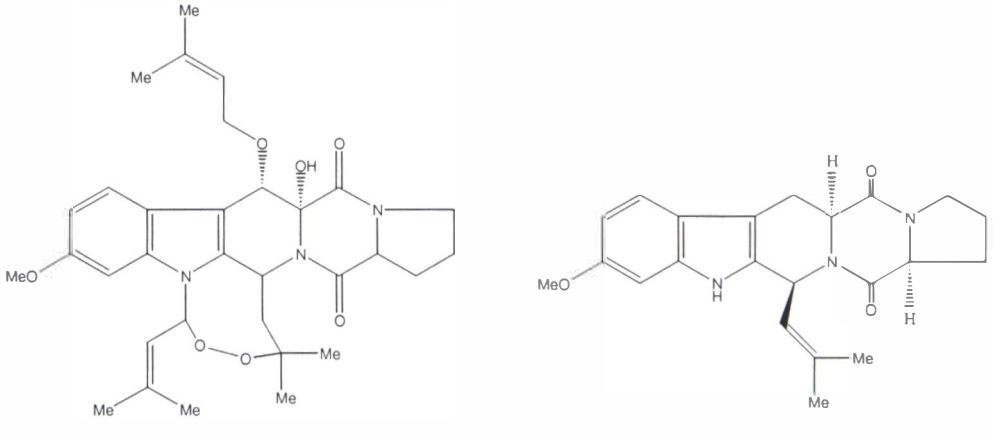


Figure 6.3 Structure of sterigmatocystin

Fumitremorgins

These metabolites (Fumitremorgins A to N)[210-212] are a series of diketopiperazines produced by certain strains of *A. fumigatus*.[213]



Fumitremorgin A Fumitremorgin C

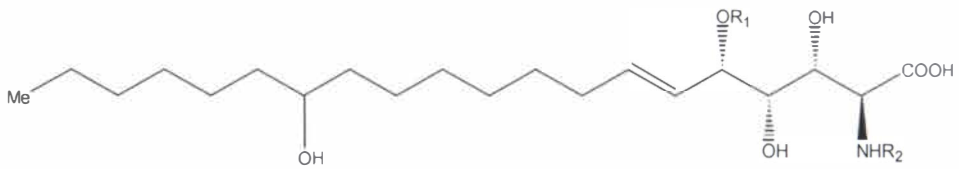
Figure 6.4 Structures of fumitremorgins

Fumitremorgins A (Figure 6.4) and B were first isolated in 1971[214-216] as tremorgenic mycotoxins since they caused severe tremor and convulsions in experimental animals due to neurotoxic properties.[217,218] In 1998, Fumitremorgin C (Figure 6.4) was found

to be effective in treating MDR (multi-drug resistance) which is a major problem in cancer chemotherapy.[219] Thereafter interest in this class of compounds led to the synthesis of a series of analogs.[220,221]

Sphingofungins

Sphingofungins were isolated from the cultivation of *Aspergillus fumigatus* (ATCC 20857) by the Merck group in 1992.[222] These compounds have four consecutive chiral centers and a *trans* olefinic group in their polar head moiety (Figure 6.5). They were found to inhibit the biosynthesis of sphingolipids due to their activity as serinepalmitoyl transferase inhibitors.[223] Sphingofungins A, B, C and D showed a limited spectrum of antifungal activity but were especially effective against *Cryptococcus* species.[222]



	R_1	R_2
Sphingofungin A	H	$C(NH)NH_2$
Sphingofungin B	H	H
Sphingofungin C	Ac	H
Sphingofungin D	H	Ac

Figure 6.5 Structures of sphingofungins

6.2 Taxonomy of *Aspergillus* sp. (A151)

In this present work, the producing organism, strain No. A151, was isolated from saline lake sand in western Australia. The identification of strain A151 was made as described by Pitt and Hocking.[107]

Morphological and cultural characteristics: Colonies on Czapek yeast extract agar (CYA) 48-55 mm in diameter (7 days, 25 °C), wrinkled, dense and velutinous, white at first and becomes green with the forming of conidial heads, reverse pale. Colonies on malt extract agar (MEA) 50-61 mm in diameter (7 days, 25 °C), similar to those on

CYA but less dense and with conidia in duller colours, reverse greenish. Colonies on 25% glycerol nitrate agar (G25N) 8-10 mm diam (7 days, 25 °C). No growth at 5 °C. Growth at 37 °C is exceptionally rapid, colonies on CYA 29-35mm in diameter in 2 days. This strain can grow at 50 °C.

Conidial apparatus develops as erect conidiophores. Tips of conidiophores enlarge and form vesicles with many phialides producing conidia in long dry chains. Conidial heads are compactly columnar, 33-47 µm in diameter, green to dark green. Conidiphores are unbranched, smooth and pale green, stipes 210-330 µm x 4-5 µm in size. Vesicles are flask-shaped, 14-25 µm in diameter, fertile over half or more of the enlarged area, bearing phialides only, the lateral ones characteristically bent so that the tips are approximately parallel to the stipe axis. Phialides crowded, pale green, 6-8 µm long. Conidia are globose to subglobose, roughened, green in mass and 2 – 3 µm in diameter.

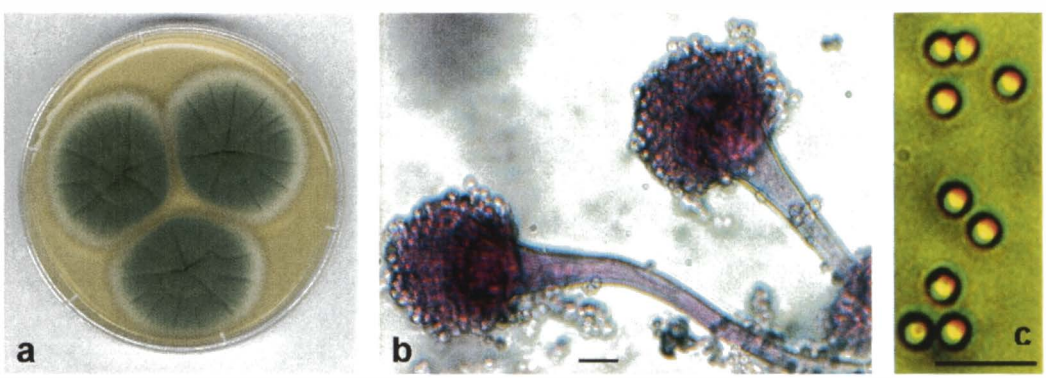


Figure 6.6 *Aspergillus fumigatus* (a) colonies on CYA (7 days, 25 °C); (b) conidiophores, stained with 0.025% lactofuchsin; (c) conidia. Bars = 10 µm

The characteristics of strain A151 are consistent with the descriptions of *Aspergillus fumigatus* given by Pitt and Hocking,[107] Kozakiewicz,[224] and Samson.[194]

6.3 Culturing and Isolation

6.3.1 Culturing and extraction

Aspergillus fumigatus (CANU A151) was grown in a liquid medium of half strength potato dextrose broth at 26 °C. Two batches were cultured. The first batch (big broth I) was cultured for four weeks and the second batch (big broth II) was cultured for eleven weeks. Each culture was filtered under suction and the mycelium and filtrate extracted separately with EtOAc. The EtOAc extracts were then evaporated under reduced pressure. The extracts were assayed for activity against the P388 murine leukaemia cell line. The filtrate extract from big broth I and the mycelial extract from big broth II showed significant activity while the filtrate extract from big broth II and the mycelial extract from big broth I exhibited very weak activity (Table 6.1). The HPLC profiles of these 4 extracts were also different from one another. This result indicated that the length of the culture period had a great influence on the secondary metabolites produced by this strain of *Aspergillus fumigatus*. The two active extracts were selected for chromatography.

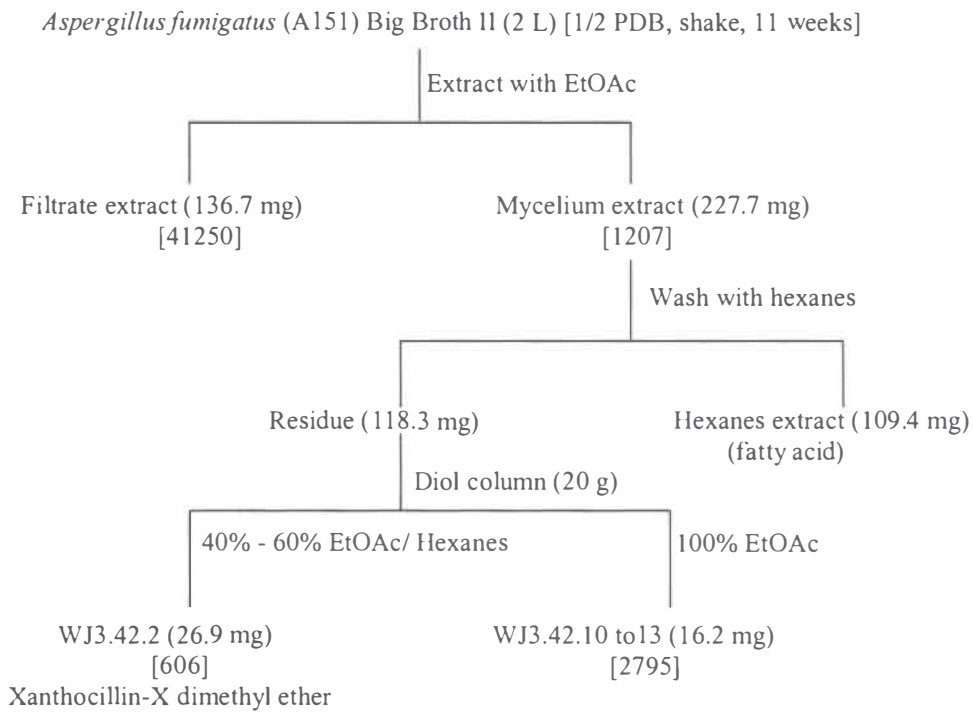
Table 6.1 Yield and activity of extracts from *Aspergillus fumigatus* (A151)

	Big Broth I (5 L, 4 weeks)		**Big Broth II (2 L, 11 weeks)**	
	Filtrate Extract	Mycelial Extract	Filtrate Extract	Mycelial Extract
Yield	590.9 mg	932.1 mg	136.7 mg	227.7 mg
P388 activity (IC_{50})	2189 ng/mL	23351 ng/mL	41250 ng/mL	1207 ng/mL

6.3.2 Chromatography of the active extract from big broth II

An outline of the isolation is illustrated in **Scheme 6.1**.

Scheme 6.1



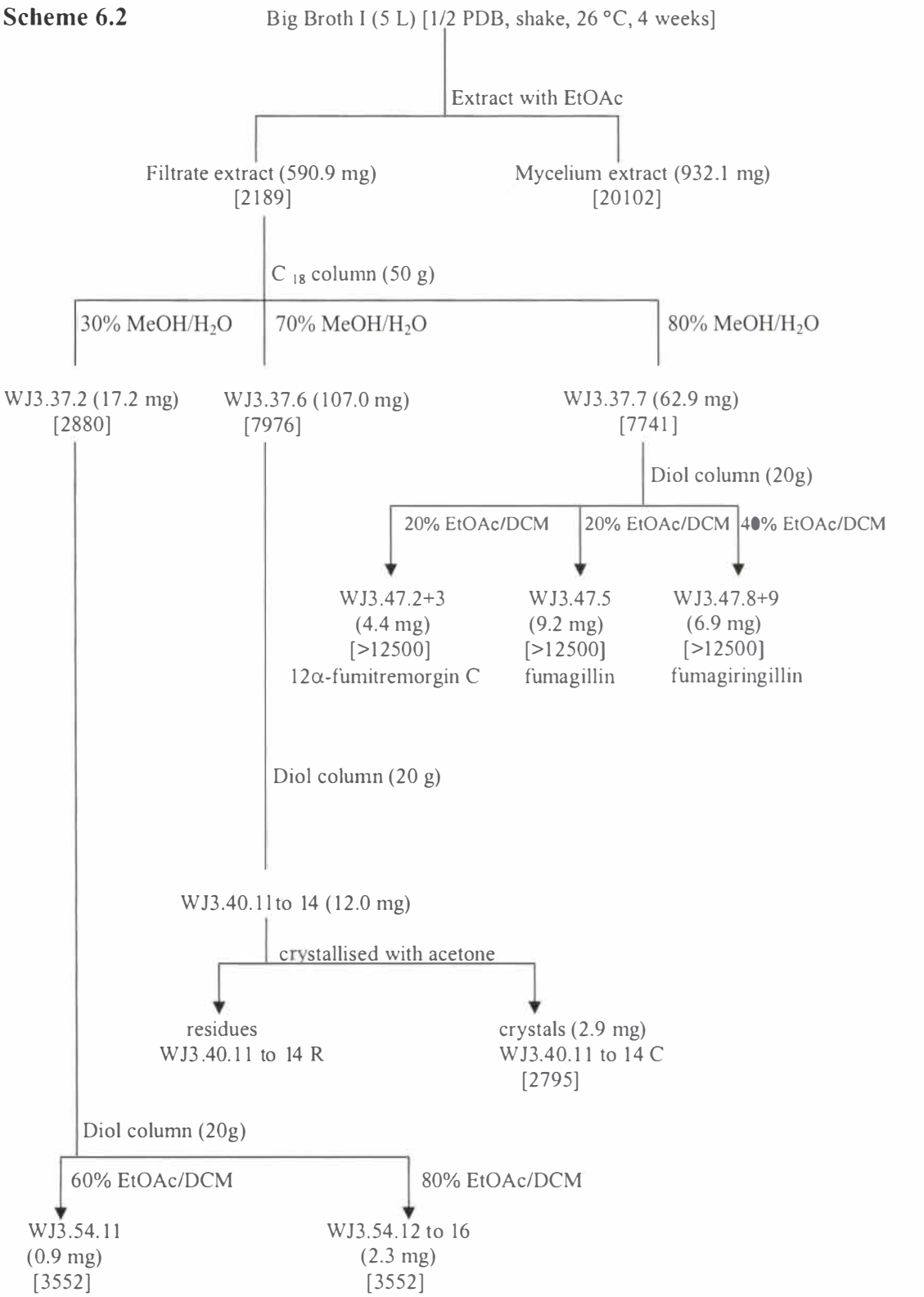
[ ]: P388 activity (IC_{50} = ng/mL)

The mycelial extract from big broth II was washed with hexanes three times to remove fatty acids. The residue (118.3 mg) was applied to a Diol (normal-phase) column, eluted with a stepped gradient from hexanes to EtOAc and then to MeOH. Seventeen fractions were collected. Fraction WJ3.42.2, eluted with 40-60% EtOAc/hexanes was a pure compound which showed strong activity against the P388 murine leukaemia cell line (IC_{50} = 606 ng/mL). The structural elucidation of the active compound WJ3.42.2 is discussed in section **6.4** . Fractions WJ3.42.10 to 13 showed the same ^{1}H NMR and HPLC profile to that of WJ3.40.11 to 14. For further purification see section **6.3.3** (Fraction 3.37.6, then subfraction WJ3.40.11 to 14).

6.3.3 Chromatography of the active extract from big broth I

An outline of the isolation is illustrated in **Scheme 6.2**.

Scheme 6.2



[ ]: P388 activity (IC_{50} = ng/mL)

The filtrate extract (590.9 mg) of big broth I was separated on a C_{18} (50 g) column, eluted with a stepped gradient from 10% MeOH/H_2O to MeOH and then CH_2Cl_2. Fifteen fractions were collected. Fractions WJ3.37.2, 6 and 7 were active against the P388 cell line (IC_{50} = 2880, 7976 and 7741 ng/mL respectively).

Fraction WJ3.37.7 (62.9 mg) was further chromatographed on 20 g of Diol, using CH_2Cl_2/hexanes (50% to 100%), EtOAc/CH_2Cl_2 (20% to 100%) and MeOH/EtOAc (50% to 100%) as the eluent. Twenty eight fractions were collected and their combination based mainly on comparisons of ^1H NMR spectra. Subfractions WJ3.47.2+3, WJ3.47.5 and WJ3.47.8+9, eluted with 20 - 40% EtOAc/CH_2Cl_2, afforded three pure compounds (fumitremorgin C, 4.4 mg; fumagillin 9.2 mg; fumagiringillin, 6.9 mg). In the P388 assay, all three pure compounds showed no notable activity (IC_{50} >12500 ng/mL). So all the other subfractions from WJ3.37.7 were submitted for P388 assay, but again, no activity was detected in those fractions. Because the mass recovered from subfractions WJ3.47.x was very close to that of the crude one, absorption of active compounds on the Diol column does not seem possible. The loss of activity might be due to decomposition of active component (s) or that the activity of fraction WJ3.37.7 was a synergistic effect of inactive components.

Fraction WJ3.37.6 (107.0 mg) was subjected to further purification using Diol (normal phase) column. The chromatography was carried out using a hexanes – DCM – EtOAc – MeOH stepped gradient. Subfraction WJ3.40.11 to 14, eluted with 40-60% EtOAc/DCM, showed biological activity (P388, IC_{50} = 2795 ng/mL). Due to the poor solubility of this fraction in all the solvents available, crystallisation was used for purifying the active compound(s). White crystals formed in acetone. Also because of the poor solubility of the crystals in all the solvents available for NMR spectroscopy, the information obtained from ^{13}C NMR and CIGAR spectra is very limited. No structural identification was achieved.

Fraction WJ3.37.2 (17.2 mg) was further chromatographed using normal phase (Diol) flash chromatography. The column was eluted using a 50% DCM/hexanes – DCM – EtOAc – MeOH stepped gradient. The fractions were combined based mainly on ^1H NMR spectroscopic analysis. Subfractions WJ3.54.11 and 12 to 16, eluted with 60%

and 80% EtOAc/DCM respectively, showed activity against the P388 cell line (IC_{50} = 3552, 3552 ng/mL, respectively). Analysis of the ^{1}H NMR spectra identified that the major components in these active fractions were simple benzenoid compounds closely related to what had been isolated previously from the extract of *Phoma* sp (K31) (see **Chapter 4**). Due to the relatively simple chemical structure and the low yield at this stage of purification, the compounds responsible for the activity in this fraction were not pursued further.

6.4 Structural Elucidation of WJ3.42.2

WJ3.42.2 was obtained as greenish-yellow needles. The HREIMS showed a peak at 316.12060 indicating the formula $C_{20}H_{16}N_2O_2$ (calcd. 316.12118). The simplicity of the NMR spectra (Table 6.2) of WJ3.42.2 was suggestive of a high degree of symmetry. In the ^{1}H NMR spectrum, one set of 1,4-disubstituted benzene protons (δ_H 6.97 and 7.78), an olefinic proton (δ_H 7.00) and methoxyl protons (δ_H 3.86) were observed. The CIGAR experiment (Table 6.2) revealed the following structural information. The methoxyl protons at δ_H 3.86 and the aromatic protons at δ_H 6.97 and 7.78 showed correlations to a quaternary aromatic carbon at δ_C 161.3. The aromatic proton at δ_H 6.97 also correlates to another quaternary aromatic carbon at δ_C 125.0. Furthermore, long-range correlations were observed from δ_H 7.00 to δ_C 131.9 and 116.4, and from δ_H 7.78 to δ_C 127.6. The isocyano group was deduced from the quaternary ^{13}C NMR signals at δ_C 173.5 which showed no correlations in the CIGAR spectrum, and by the molecular formula of WJ3.42.2. Accordingly, compound WJ3.42.2 contains partial structure I (Figure 6.7).

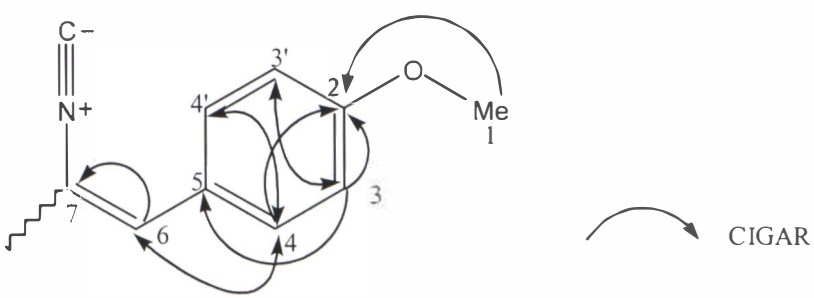


Figure 6.7 Partial structure I of WJ3.42.2

On the basis of the molecular weight there must be two units of partial structure I. Therefore, the structure of compound WJ3.42.2 was suggested as shown in Figure 6.8.

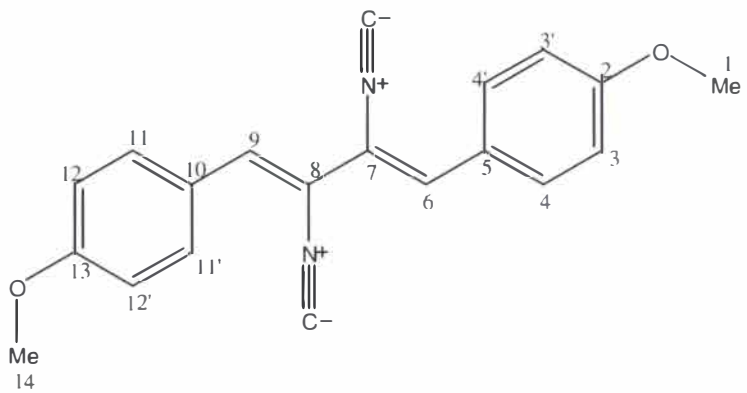


Figure 6.8 Structure of WJ3.42.2 (xanthocillin-X dimethyl ether)

The relative stereochemistry of compound WJ3.42.2 was finally determined by a single-crystal X-ray diffraction analysis. A computer-generated perspective drawing of WJ3.42.2 is shown in Figure 6.9.

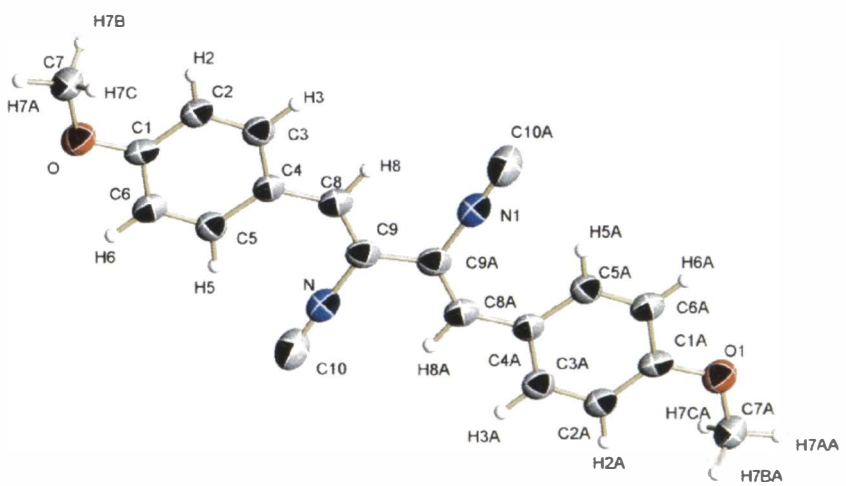


Figure 6.9 Structure of WJ3.42.2 determined by X-ray crystallographic study

From a literature search, compound WJ3.42.2 was found to be identical to xanthocillin-X dimethyl ether.[225-227]

Table 6.2 ^{1}H, ^{13}C, HSQC, COSY and CIGAR NMR data for WJ3.42.2[a]

no.	$^{13}C\ \delta$[c]	$^{1}H\ \delta$[b]	COSY	CIGAR[d]	
				H ⟶ C	C ⟶ H
1, 14	55.7 (CH_3)	3.86 (3H, s)		161.3	
2, 13	161.3 (C)				3.86, 6.97, 7.78
3, 3', 12, 12'	114.7 (CH)	6.97 (2H, d, 9.0)	7.78	114.7(w), 125.0, 161.3	6.97(w)
4, 4', 11, 11'	131.9 (CH)	7.78 (2H, d, 9.5)	6.97	127.6, 131.9, 161.3	7.00, 7.78
5, 10	125.0 (C)				6.97
6, 9	127.6 (CH)	7.00 (1H, s)		131.9, 116.4	7.78
7, 8	116.4 (C)				7.00
7-NC, 8-NC	173.5 (C)				

[a] ^{1}H NMR spectra recorded at 500 MHz in $CDCl_3$, ^{13}C NMR spectra recorded at 125 MHz in $CDCl_3$.
[b] ^{1}H chemical shift values (δppm from $SiMe_4$) followed by number of protons, multiplicity and coupling constant (J/Hz).
[c] primary, secondary, tertiary and quaternary carbons, assigned by APT.
[d] long range ^{1}H-^{13}C correlation from H to C or C to H observed in the CIGAR experiment.

Biological Activity:

Biological testing of xanthocillin X dimethyl ether (WJ3.42.2) in this study showed it to have significant cytotoxicity against the P388 murine leukaemia cell line (IC_{50} 606 ng/mL). Other activities have also been reported in the literature: xanthocillin dimethyl ether inhibited Newcastle disease, *Herpes simplex*, and vaccinia viruses, and also inhibited the growth of *Bacillus subtilis*.[228] Antitumour activity was shown in *in vitro* and *in vivo* mouse tests with LD_{50} being >120 mg I/kg in mice. Tumour growth in humans is inhibited by the administration of xanthocillin X dimethyl ether in the range 100-3000 mg/day for an average adult patient.[229]

6.5 Structural Elucidation of WJ3.47.2+3

WJ3.47.2+3 was obtained as a pale yellow powder. The molecular formula was determined to be $C_{22}H_{25}N_3O_3$ [Found 380.1975 (MH^+), calcd: 380.1974] by HRESMS measurement.

In the ^1H NMR spectrum, WJ3.47.2+3 showed signals due to an N-H proton (δ_H 7.79, br s, H-1) and a 1,2,4-trisubstituted benzene ring (δ_H 7.43, d, J=9.0 Hz, H-16; δ_H 6.81, dd, J=8.0, 2.0 Hz, H-17 and δ_H 6.85, d, J=1.5 Hz, H-19) being part of a 2,3,6-substituted indole system, and an olefinic proton (δ_H 4.90, dd, J=10.0, 1.5 Hz, H-21), a methoxy (δ_H 3.83, s, 18-OCH_3) and two methyl (δ_H 1.64, d, J=1.0 Hz, H-23 and δ_H 1.99, d, J=1.5 Hz, H-24) groups along with signals due to several methine and methylene groups (Table 6.3). The ^{13}C NMR spectrum of WJ3.47.2+3, analyzed by the APT method, indicated the presence of two amide carbonyls (δ_C 169.7, C-5 and δ_C 165.9, C-11), an oxygen-bearing aromatic carbon (δ_C 156.7, C-18) and a methoxy (δ_C 56.0, 18-OCH_3), two methyl (δ_C 18.3, C-24 and δ_C 25.9, C-23) groups and four methylene (δ_C 22.1, C-13; δ_C 23.2, C-8; δ_C 28.8, C-7 and δ_C 45.6, C-9) groups together with seven methines and five quaternary carbons (Table 6.3).

A close inspection and interpretation of the ^1H and ^{13}C NMR spectra of WJ3.47.2+3 by HSQC and COSY experiments (Table 6.3) led to the partial structures as shown in bold in Figure 6.10.

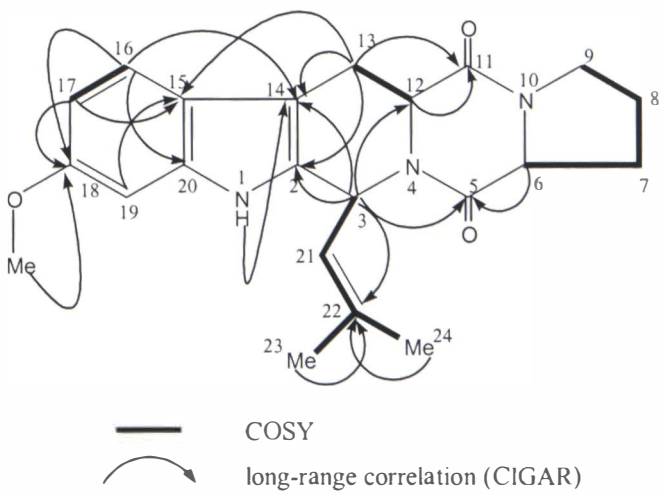


Figure 6.10 Planar structure for WJ3.47.2+3

The CIGAR spectrum was then obtained in order to determine the total structure of WJ3.47.2+3. In the indole system (Figure 6.10), the benzene protons H-16 (δ_H 7.43), H-17 (δ_H 6.81) and the methoxy protons at δ_H 3.83 showed long-range correlations to the oxygen-bearing quaternary carbon at δ_C 156.7 (C-18), while H-17 and H-19 (δ_H 6.85) and H-16 showed long-range correlations to the quaternary carbons at δ_C 120.9 (C-15) and at δ_C 137.2 (C-20), respectively. Therefore, C-18 (δ_C 156.7), C-15 (δ_C 120.9) and C-20 (δ_C 137.2) could be assigned. The other two quaternary carbons in the indole system, C-2 and C-14, were assigned based on the long-range correlations of H-16, N-H (δ_H 7.79) and the methylene protons at δ_H 3.09 (H-13a) and 3.51 (H-13b) to δ_C 106.5 (C-14) and H-13 and H-3 (δ_H 5.98) to δ_C 132.3 (C-2). The correlation peaks observed between H-3 and H-6 (δ_H 4.11) to δ_C 169.7 (C-5) and between H-13a and H-12 (δ_H 4.18) to δ_C 165.9 (C-11) led to the assignment of the two amide carbonyls. The assignment of C-22 was suggested by the long-range correlations between H-23 (δ_H 1.64), H-24 (δ_H 1.99) and H-3 to the quaternary carbon at δ_C 134.2.

Finally, the connectivities were determined by extensive analysis of the CIGAR spectrum. As shown in Figure 6.10, both H-13a and H-13b showed long-range correlations to the carbons C-14 (δ_C 106.5) and C-2 (δ_C 132.3), and also to C-11 (δ_C 165.9) and C-15 (δ_C 120.9), respectively. This allowed C-14 and C-11 to be connected through C-13 and C-12. The correlation peaks between H-3 to C-12, C-2, C-14 and C-5 led to the connection of C-3 to N-4 and then to C-12, C-3 to C-2 and C-3 to N-4 and then to C-5 respectively. The connectivity of C-5 and C-6 was determined by the long-range correlation of H-6 to C-5. The formation of the five membered heterocyclic ring by connection of C-6 and C-9 across the nitrogen atom at position 10 was supported by the chemical shift values of C-6 (δ_C 59.4) and C-9 (δ_C 45.6). Thus, the planar structure of WJ3.47.2+3 was deduced (Figure 6.10). From a literature search, this compound was found to be identical to fumitremorgin C, previously isolated from *A. fumigatus* in 1977.[230]

Table 6.3 ^{1}H, ^{13}C, HSQC, COSY and CIGAR NMR data for WJ3.47.2+3[a]

no.	^{13}C δ[c]	^{1}H δ[b]	COSY	CIGAR[d]
1		7.79 (1H, br s, N-H)		106.5, 120.9
2	132.3 (C)			
3	51.2 (CH)	5.98 (1H, d, 9.5)	4.90	57.0, 106.5, 124.3, 132.3, 134.2, 169.7
5	169.7 (C)			
6	59.4 (CH)	4.11 (1H, t, 8.0)	2.23, 2.40	28.8, 169.7
7	28.8 (CH_2)	H7a: 2.23 (1H, m)	4.11, 2.40, 2.05	
		H7b: 2.40 (1H, m)	4.11, 2.23	45.6
8	23.2 (CH_2)	H8a: 1.94 (1H, m)	2.05, 2.40, 3.63	
		H8b: 2.05 (1H, m)	1.94, 2.23, 3.63	
9	45.6 (CH_2)	3.63 (2H, m)	1.94, 2.05	
11	165.9 (C)			
12	57.0 (CH)	4.18 (1H, dd,11.5, 5.0)	3.09, 3.51	165.9
13	22.1 (CH_2)	H13a: 3.09 (1H, dd,16.0, 11.5)	4.18, 3.51	57.0, 106.5, 132.3, 165.9
		H13b: 3.51 (1H, dd,16.0, 5.5)	4.18, 3.09	57.0, 106.5, 120.9, 132.3
14	106.5 (C)			
15	120.9 (C)			
16	119.1 (CH)	7.43 (1H, d, 9.0)	6.81	106.5, 137.2, 156.7
17	109.7 (CH)	6.81 (1H, dd, 8.0, 2.0)	7.43	95.4, 120.9, 156.7
18	156.7 (C)			
18-OMe	56.0 (CH_3)	3.83 (3H, s)		156.7
19	95.4 (CH)	6.85 (1H, d, 1.5)		109.7, 120.9, 156.7
20	137.2 (C)			
21	124.3 (CH)	4.90 (1H, dd,10.0, 1.5)	1.64, 1.99, 5.98	18.3, 25.9
22	134.2 (C)			
23	25.9 (CH_3)	1.64 (3H, d, 1.0)	4.90	18.3, 124.3, 134.2
24	18.3 (CH_3)	1.99 (3H, d, 1.5)	4.90	25.9, 124.3, 134.2

[a] ^{1}H NMR spectra recorded at 500 MHz in $CDCl_3$, ^{13}C NMR spectra recorded at 125 MHz in $CDCl_3$.
[b] ^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by number of protons, multiplicity and coupling constant (J/Hz).
[c] primary, secondary, tertiary and quaternary carbons, assigned by APT.
[d] long range ^{1}H-^{13}C correlation from H to C observed in the CIGAR experiment.

The assignment of the stereochemistry of WJ3.47.2+3, as shown in Figure 6.11, was based on the comparison of the ^{1}H and ^{13}C NMR data with that of the two isomers of fumitremorgin C. They had previously been synthesized by Hino *et al.*[231] This determination was also supported by the optical rotation of WJ3.47.2+3 which showed $[\alpha]_D$ −11.9 ° in MeOH (c 0.53) at 20 °C (12α–fumitremorgin C showed $[\alpha]_D$ − 13 ° (c 0.53, MeOH) while 12β–fumitremorgin C has a rotation of +239 ° (c 0.11, MeOH) at 28 °C.[231]

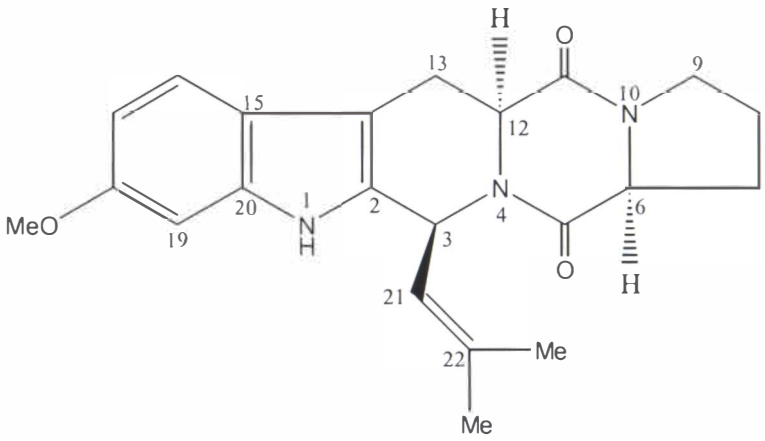


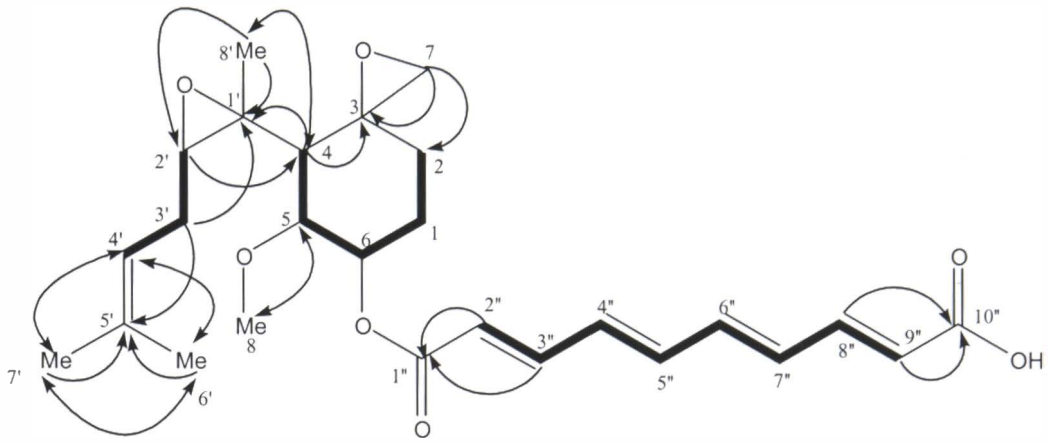
Figure 6.11 Structure for WJ3.47.2+3 (12α–fumitremorgin C)

Biological Activity:

No activity was exhibited in cytotoxicity or antimicrobial assays even though fumitremorgin C was reported to be a tremorgenic mycotoxin as early as 1977.[230] It was also reported that fumitremorgin C showed an inhibitory activity on the cell cycle progression of mouse tsFT210 cells in the M phase with a MIC value 4.1 μM. As products of oncogenes and tumour suppressor genes are involved in the regulation of the mammalian cell cycle, cell cycle inhibitors were thought to be good candidates for cancer chemotherapy and also to be a source for providing molecular probes useful in elucidating the regulatory mechanism of the cell cycle.[232] The other interesting activity of fumitremorgin C is that this compound can enhance the sensitivity of a mitoxantrone-selected colon carcinoma cell line, S1-M1-3.2 to various antitumour agents thereby reversing multi-drug resistance.[220]

6.6 Structural Elucidation of WJ3.47.5

WJ3.47.5 was obtained as a pale yellow powder. Analysis of the HRESMS ([M+H$^+$], m/z 459.2375, calcd. 459.2383) and the ^{13}C NMR spectrum (Table 6.4) of WJ3.47.5 led to the molecular formula $C_{26}H_{34}O_7$. The ^{13}C NMR spectrum showed all 26 carbons, which were identified as four methyls, four methylenes, four methines, nine olefinic methines, three quaternary carbons, and two carbonyl carbons by the HSQC and APT spectra (Table 6.4). The COSY spectrum (Table 6.4) of WJ3.47.5 revealed the substructures from H-2 (δ_H 1.09 & 2.10) to H-4 (δ_H 2.03) through H-1 (δ_H 1.86 & 1.98), H-6 (δ_H 5.71) and H-5 (δ_H 3.68), from H-2' (δ_H 2.64) to H-4' (δ_H 5.20) through H-3' (δ_H 2.15 & 2.37), and from H-2" (δ_H 5.99) to H-9" (δ_H 5.93) (bold lines in Figure 6.12). In the CIGAR spectrum (Table 6.4) of WJ3.47.5, the correlations of H-6' (δ_H 1.73) and H-7' (δ_H 1.64) to C-4' (δ_C 118.7) and C-5' (δ_C 135.1) and H-3' to C-5' led to the connectivity of C-6' and C-7' to C-4' through C-5' (Figure 6.12). The chemical shifts of C-1' (δ_C 59.0) and C-2' (δ_C 61.2) suggested that they are oxygen-bearing carbons. The connectivity of C-1' to C-2', and the spiroepoxide moiety was indicated by the correlation peaks observed between H-3'b (δ_H 2.37) to C-1' and the connectivity of C-8' (δ_C 14.0) to C-4 through C-1' was indicated by the correlations of H-4 and H-8' (δ_H 1.21) to C-1', H-8' to C-2', H-2' to C-4 and H-4 to C-8'. The correlations of H-7a (δ_H 2.55) to C-2 (δ_C 29.5), H-7a, H-7b (δ_H 2.99) and H-4 to C-3 (δ_C 59.7) led to the connectivity of C-2 to C-3 and then to C-4, and led to the connectivity of a spiroepoxide moiety, C-3 to C-7. The spiroepoxide was also suggested by the chemical shifts of C-3 and C-7 (δ_C 59.7 and 51.0, respectively). The methoxy group (δ_C 56.9, δ_H 3.43) was assigned based on the correlations of H-5 to C-8 (δ_C 56.9) and H-8 (δ_H 3.43) to C-5 (δ_C 79.5). Two carbonyl carbons, C-1" (δ_C 166.3) and C-10" (δ_C 170.8), were assigned by the correlations observed between H-2" (δ_H 5.99) and H-3" (δ_H 7.28) to C-1", and between H-8" (δ_H 7.33) and H-9" (δ_H 5.93) to C-10". The connectivity of C-6 to C-1" through an oxygen and C-10" to a hydroxy group were made based on the chemical shifts of C-1" and C-10" and the molecular formula of WJ3.47.5. Thus, the planar structure of WJ3.47.5 was proposed as shown in Figure 6.12. This proposed structure is identical to fumagillin, a known fungal metabolite.



COSY

CIGAR

Figure 6.12 Planar structure of WJ3.47.5

The stereochemistry of WJ3.47.5 was confirmed as shown in Figure 6.13 by literature data comparison.[233] The single crystal X-ray study[233] revealed that the cyclohexyl ring is in a chair conformation (as shown in Figure 6.13), the oxygen of the spiroepoxide ring is quasi-axial, the C-4 substituent and the methoxy group on C-5 are in equatorial positions, the decatetraenedioyl sidechain is attached through an axially orientated linkage, and all four conjugated double bonds have an E configuration.

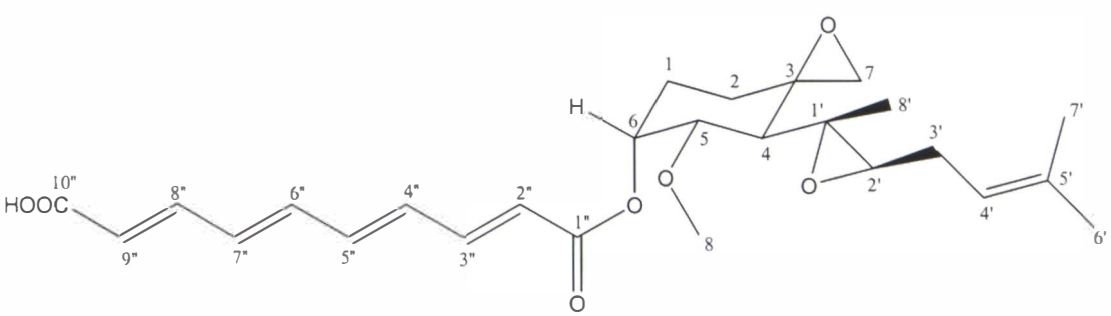


Figure 6.13 Stereostructure of WJ3.47.5 (fumagillin)

Table 6.4 ^{1}H, ^{13}C, HSQC, COSY and CIGAR NMR data for WJ3.47.5[a]

no.	^{13}C δ[c]	^{1}H δ[b]	COSY	CIGAR[d]
1	25.9 (CH_2)	H1α: 1.98 (1H, m)	1.09 (w), 1.86, 2.10, 5.71	
		H1β: 1.86 (1H, m)	1.09, 1.98, 2.10, 5.71	59.7(vw), 79.5(vw)
2	29.5 (CH_2)	H2α: 2.10 (1H, m)	1.86, 2.10	
		H2β: 1.09 (1H, m)	1.09, 1.86	25.9 (vw)
3	59.7 (C)			
4	48.3 (CH)	2.03 (1H, d, 11.0)	3.68	14.0, 59.0, 59.7, 66.6, 79.5
5	79.5 (CH)	3.68 (1H, dd, 11.0, 2.5)	2.03, 5.71	48.3, 56.9, 66.6
6	66.6 (CH)	5.71 (1H, m)	1.86, 1.98, 3.68	
7	51.0 (CH_2)	H7a: 2.55 (1H, d, 4.0)	2.99	29.5, 59.7
		H7b: 2.99 (1H, d, 3.5)	2.55	59.7
8	56.9 (CH_3)	3.43 (3H, s)		79.5
1'	59.0 (C)			
2'	61.2 (CH)	2.64 (1H, t, 6.5)	2.15, 2.37	27.5, 48.3, 59.0(vw), 118.7
3'	27.5 (CH_2)	H3'a: 2.15 (1H, m)	2.37, 2.64, 5.20	59.0(w), 61.2, 118.7, 135.1
		H3'b: 2.37 (1H, m)	2.15, 2.64, 5.20	59.0, 61.2, 118.7, 135.1
4'	118.7 (CH)	5.20 (1H, m)	1.64, 1.73, 2.15, 2.37	18.2, 25.9, 27.5
5'	135.1 (C)			
6'	25.9 (CH_3)	1.73 (3H, s)	5.20	18.2, 61.2, 118.7, 135.1
7'	18.2 (CH_3)	1.64 (3H, s)	5.20	25.9, 61.2, 118.7, 135.1
8'	14.0 (CH_3)	1.21 (3H, s)		48.3, 59.0, 61.2
1"	166.3 (C)			
2"	123.6 (CH)	5.99 (1H, d, 15.5)	7.28	134.3, 166.3
3"	143.7 (CH)	7.28 (1H, dd, 14.5, 11.0)	5.99, 6.43	166.3
4"	134.3 (CH)	6.43 (1H, m)	6.60, 7.28	140.0
5"	140.0 (CH)	6.60 (1H, m)	6.43	133.5, 143.7
6"	139.0 (CH)	6.59 (1H, m)	6.47	134.3, 145.4
7"	133.5 (CH)	6.47 (1H, m)	6.59, 7.33	139.0
8"	145.4 (CH)	7.33 (1H, dd, 15.5, 11.5)	5.93, 6.47	170.8
9"	122.4 (CH)	5.93 (1H, d, 15.0)	7.33	133.5, 170.8
10"	170.8 (C)			

[a] ^{1}H NMR spectra recorded at 500 MHz in $CDCl_3$, ^{13}C NMR spectra recorded at 125 MHz in $CDCl_3$.
[b] ^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by number of protons, multiplicity and coupling constant (J/Hz).
[c] primary, secondary, tertiary and quaternary carbons, assigned by APT.
[d] long range ^{1}H-^{13}C correlation from H to C observed in the CIGAR experiment.

Biological Activity of Fumagillin:

P388 Activity: IC_{50} >12500 ng/mL.

Antimicrobial activity: 3 mm zone of inhibition against *Bacillus subtilis* at a concentration of 60 μg/disk.

Antiviral activity: CYT >+, TYP 4 for *Polio virus* at a concentration of 40 μg/disk.

The anticancer activity of fumagillin has long been recognised[234] though no activity against the P388 cell line was exhibited in assays in this present work. As a potent angiogenesis (new blood vessel formation) inhibitor fumagillin suppresses the growth of a wide variety of tumours,[235,236] but because of toxic side-effects it cannot be utilised. Derivatives with less side-effects have been synthesized, and one of them (TNP-470, Figure 6.14) is now undergoing clinical trials for the treatment of a variety of cancers.[237,238] Fumagillin and its synthetic analogue TNP470 (Figure 6.14) are currently the only treatment against cryptosporidiosis and against microsporidiosis caused by *Enterocytozoon bieneusi*, which are frequent and fatal in AIDS patients.[239,240] Fumagillin has also acquired importance in veterinary medicine against microsporidiosis of bees and fish.[241-243] It is a strong antiparasitic and amoebicidal compound[244] and one of two chemotherapeutics capable of controlling proliferative kidney disease of salmonid fish infected by an unclassified myxosporean parasite, the so-called 'PKX'.[245]

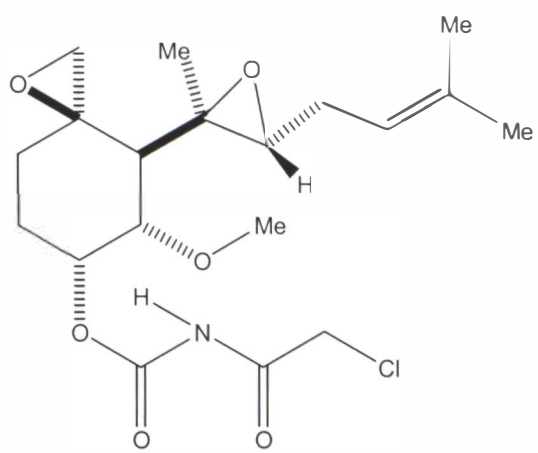


Figure 6.14 Structure of TNP-470

6.7 Structural Elucidation of WJ3.47.8+9

WJ3.47.8+9 was also obtained as a pale yellow powder. The molecular formula was deduced from the HRESMS as $C_{26}H_{36}O_8$, showing an accurate mass at *m/z* 475.2343 [(M-H^+), calcd. 475.2332]. The ^1H (Figure 6.19) and ^{13}C NMR spectra (Table 6.5) of WJ3.47.8+9 and those of WJ3.47.5 (fumagillin, Table 6.4) were very similar, except for the chemical shifts of four oxygen-bearing carbons (δ_C 51.0, 59.0, 59.7 and 61.2) in the two spiroepoxide rings of fumagillin which were shifted down field (δ_C 77.1, 78.7, 81.0 and 85.3) in WJ3.47.8+9. The ^{13}C NMR spectrum of WJ3.47.8+9 showed twenty six signals, which could be classified, by the APT spectrum, as thirteen methine, four methylene, four methyl, and five quaternary carbons. The assignment of proton(s) attached to their corresponding carbons was readily accomplished by the HSQC technique (Table 6.5). The COSY spectrum (Table 6.5) of WJ3.47.8+9 revealed the connectivity from H-9 (δ_H 2.19) through H-1 (δ_H 3.68), H-2 (δ_H 5.67), H-3 (δ_H 1.93, 2.07) to H-4 (δ_H 1.63, 1.71), from H-10 (δ_H 3.37) to H-11 (δ_H 2.09, 2.39) and then to H-12 (δ_H 5.29), and from H-2' to H-9' (bold line, Figure 6.15). The CIGAR spectral data (Table 6.5, Figure 6.20) of WJ3.47.8+9 demonstrated the following information: the connectivity of the methoxy group (δ_C 57.1, δ_H 3.39) to C-1 (δ_C 76.9) was determined by the correlations of H-1 to C-17 and H-17 to C-1; the connectivity of C-4 (δ_C 27.9) to C-9 (δ_C 54.1) through C-5 (δ_C 81.0) was deduced by the correlation peaks between H-4β (δ_H 1.71) and H-1 to C-5 and between H-4β to C-9; The correlation peaks between H-6a (δ_H 3.81) and H-6b (δ_H 3.65) to C-5 and H-6a to C-9 allowed the connection of C-5 to C-6 (δ_C 77.1); The connectivity of C-8 and C-6 through oxygen was assumed by their chemical shifts (δ_C 85.3 and 77.1, respectively) and supported by the correlation of H-6a to C-8; The correlations observed between H-9 and H-10 (δ_H 3.37) to C-14 (δ_C 15.7) and H-9, H-10 and H-14 to C-8 led to the connection of C-14 to C-8 then to C-9, and C-14 to C-8 then to C-10. The connectivity of C-16 (δ_C 26.1) and C-15 (δ_C 18.1) to C-12 (δ_C 121.9) through C-13 (δ_C 133.3) was deduced by the correlations between H-12 and H-15 (δ_H 1.64) to C-16, between H-12 and H-16 (δ_H 1.71) to C-15, and between H-11a, H-11b, H-15 and H-16 to C-13. The connectivity of the two carbonyl carbons with C-2' and C-9'

was made based on the correlations of H-2' (δ_H 5.98) and H-3' (δ_H 7.30) to C-1' (δ_C 166.3) and H-8' (δ_H 7.36) and H-9' (δ_H 5.95) to C-10' (δ_C 170.7). The assignment of C-1' and C-10' were deduced by comparison of the chemical shifts of WJ3.47.8+9 with those of fumagillin.[233] The IR spectrum of compound WJ3.47.8+9 showed absorptions at 1706 and 1627 cm^{-1}, confirming the presence of carbonyl functionalities in WJ3.47.8+9. The planar structure was determined as shown in Figure 6.15. Compound WJ3.47.8+9 is a new compound and has been named fumagiringillin.

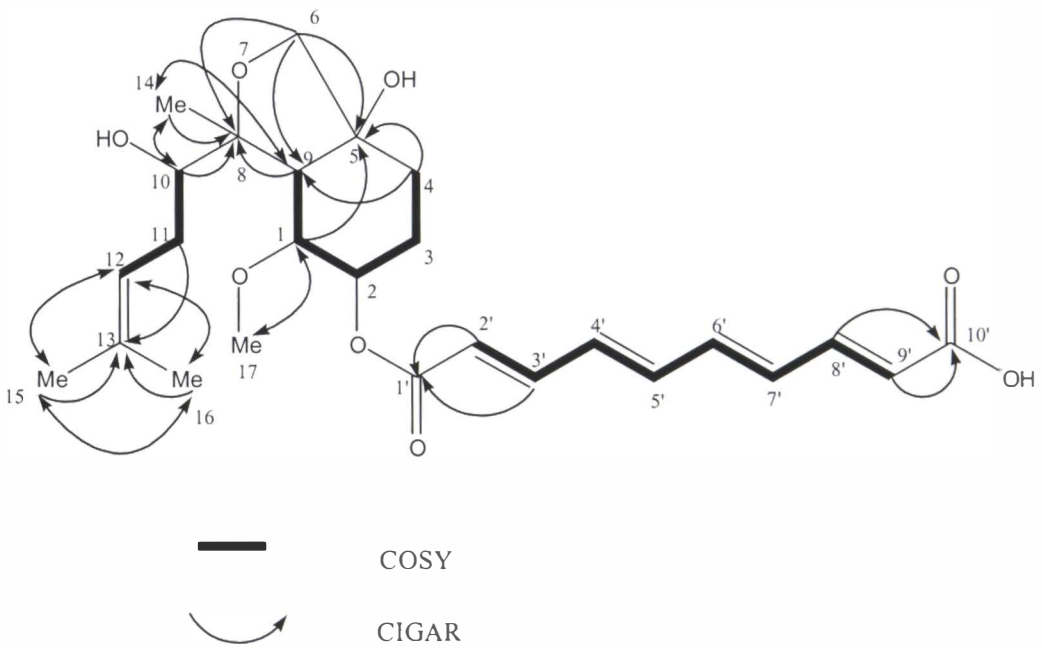


Figure 6.15 The planar structure of WJ3.47.8+9

The relative stereochemistry of WJ3.47.8+9 was assigned on the basis of coupling constants and a 2D NOESY (Table 6.5) experiment. The protons of the cyclohexyl ring are denoted α or β, indicating their positions below or above the reference plane of the cyclohexyl ring. The large coupling constant between H-1 and H-9 (12.0 Hz) and the small coupling constant between H-2 and H-1 (3.0 Hz) indicated that H-1 and H-9 were axial while H-2 was equatorial. A chair conformation of the six-membered ring as shown in Figure 6.17 was suggested. Therefore, the methoxy group on C-1 is equatorial while the decatetraenedioyl side chain is axial. An NOE correlation between the proton at C-1 and the methyl protons at C-8, clearly position these groups

on the same side of the molecule (Figure 6.16). The NOE correlation between H-12 and H-16 indicated that H-12 and H-16 were in a *cis* relationship while H-12 and H-15 were in a *trans* arrangement. The assignment of stereochemistry across the ring junction was guided by the observation of a positive NOE correlation between H-4α and H-9. This correlation allowed the assignment of *trans* stereochemistry to be made. The two possible stereoisomers were modelled using the Chem 3D programe. After energy minimisation the H-9/H-4 proton distances were 3.931 and 4.250Å for the *cis* isomer while for the *trans* isomer the inter-proton distances were 2.783 and 3.677Å. This assignment was also supported by the measurement of the spatial distance between H-3 and H-6. If 5-OH was in a β position, H-6 should be distal from H-3 and no NOE should be observed. If 5-OH was in an α position, the close distance (2.396 Å) between one of the protons at C-3 and one of the protons at C-6 should show a NOE correlation. No NOE correlation was observed between the C-3 and C-6 protons in the NOESY experiment. The stereochemistries of the four conjugated double bonds were determined as all-*E* based on the coupling constants ($J_{2',3'}$ =15.5 Hz, $J_{8',9'}$ =15.5 Hz) and the NOE correlations between H-2' and H-4', H-3' and H-5', H-4' and H-6', H-5' and H-7', H-6' and H-8', and H-7' and H-9' (Figure 6.16). The stereochemistry of the hydroxyl group at C-10 could not be assigned with any certainty. However, a positive NOE correlation was observed between H-9 and H-10. Together, these observations led to assignment of the relative stereochemistry shown in Figure 6.17 for WJ3.47.8+9.

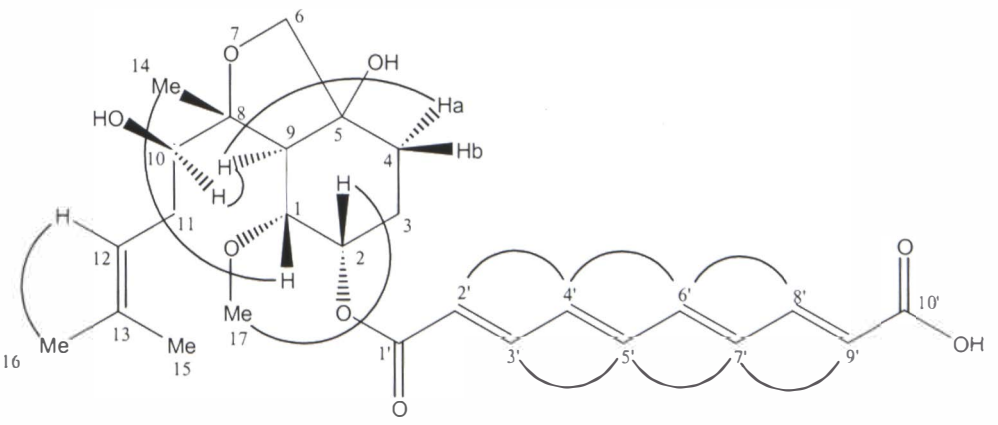


Figure 6.16 NOE correlations in NOESY spectrum of WJ3.47.8+9

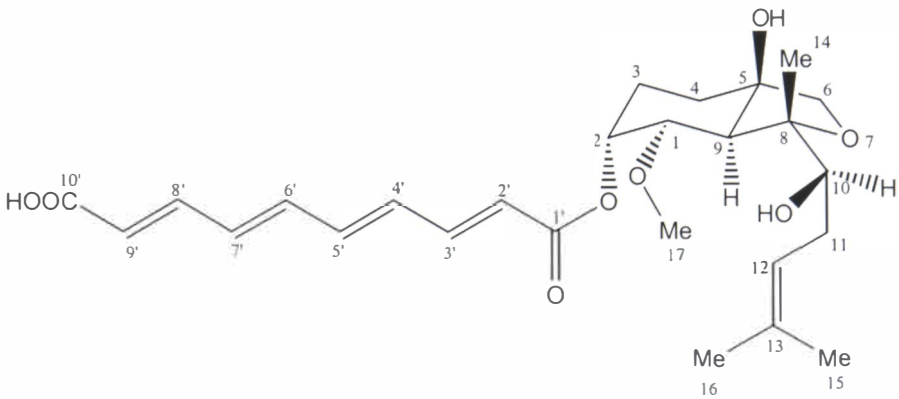


Figure 6.17 Stereostructure of WJ3.47.8+9

By checking the retention times and UV spectra in the HPLC profiles of the pure compound WJ3.47.8+9 and the crude extract (filtrate extract of big broth I), it was determined that WJ3.47.8+9 originally existed in the crude extracts.

Mechanism of the formation of WJ3.47.8+9:

Fumagillin was assumed to be a precursor of WJ3.47.8+9 and the formation of the latter was proposed in the following ways (Figure 6.18).

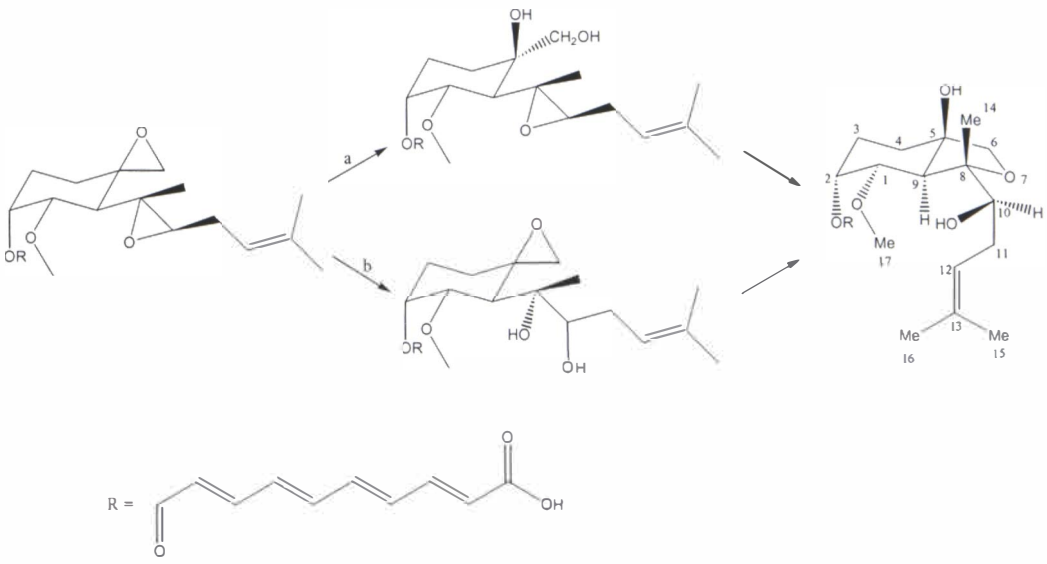


Figure 6.18 The proposed formation of WJ3.47.8+9

Biological activity of WJ3.47.8+9:

P388 activity: > 12500 ng/mL

Antimicrobial activity: none detected

6.8 Discussion

A marine strain of *Aspergillus fumigatus* (A151) was incubated in ½ PDB for different culture periods. The metabolites of 4-week broth were totally different from those of 11-week broth. It was indicated that the length of the culture period greatly influenced the production of secondary metabolites. From the mycelium of the broth cultured for eleven weeks, one biologically active compound, xanthocillin-X dimethyl ether (IC_{50} 606 ng/mL for the P388 cell line), was obtained. From the filtrate of the broth cultured for four weeks, two known compounds, 12α–fumitremorgin C and fumagillin, and a novel compound, fumagiringillin, were isolated. All three compounds showed no notable P388 activity although the crude extract exhibited moderate activity with an IC_{50} of 7741 ng/mL. The loss of activity was likely due to the decomposition of active component (s) or that the activity of crude extract was a synergistic effect of inactive components.

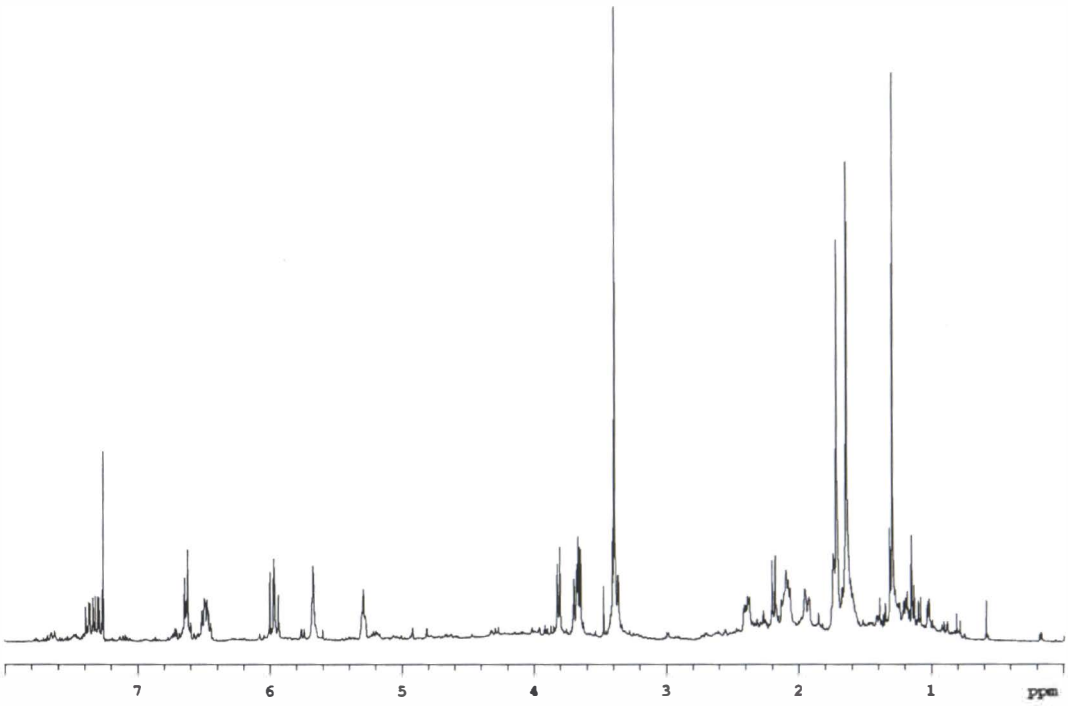


Figure 6.19 ^{1}H NMR spectrum of WJ3.47.8+9

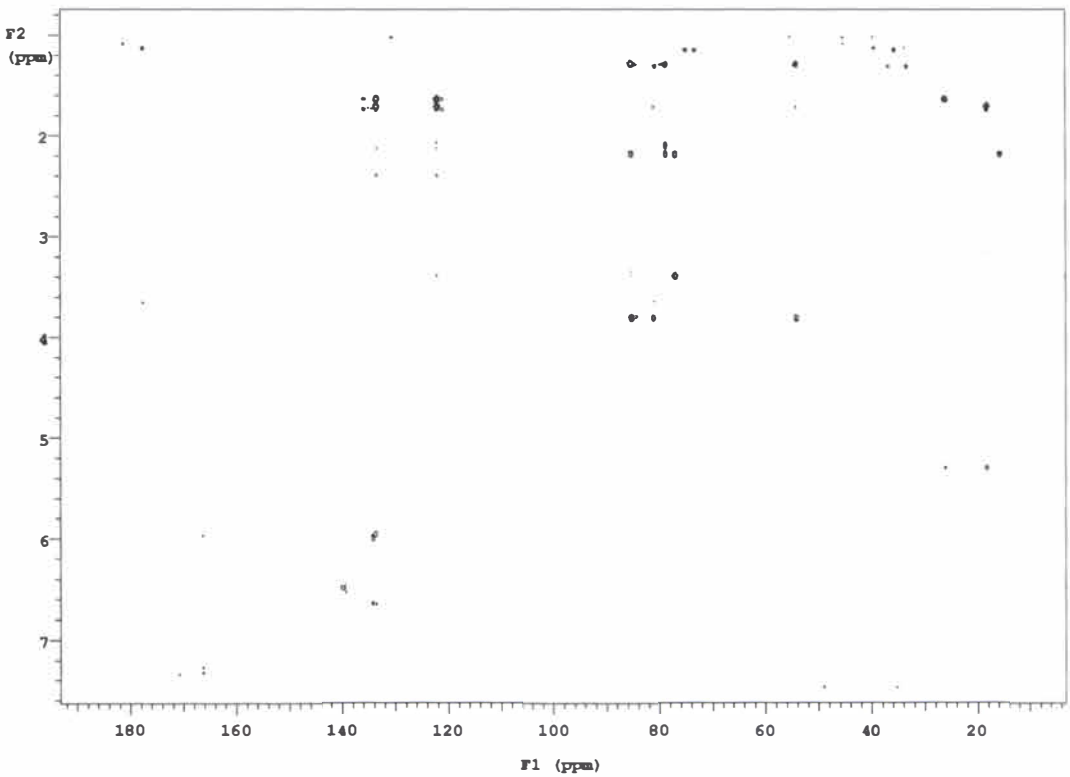


Figure 6.20 CIGAR spectrum of WJ3.47.8+9

Table 6.5 ^{1}H, ^{13}C, HSQC, COSY and CIGAR NMR data for WJ3.47.8+9[a]

no.	^{13}C δ[c]	^{1}H δ[b]	COSY	NOESY	CIGAR[d]
1	76.9 (CH)	3.68 (1H, dd, 12.0, 3.0)	2.19, 5.67	1.29, 2.19, 5.67	54.1(w), 57.1(w), 66.8(w), 81.0(w)
2	66.8 (CH)	5.67 (1H, m)	1.93, 3.68	3.39, 3.68, 1.93	
3	25.9 (CH_2)	H3β: 1.93 (1H, m)	1.63, 2.07, 5.67	1.93	
		H3α: 2.07 (1H, m)	1.63, 1.71, 1.93	2.07	
4	27.9 (CH_2)	H4α: 1.63 (1H, m)	1.93, 2.07	2.19	25.9
		H4β: 1.71 (1H, m)	1.93, 2.07	1.93	54.1, 66.8(w), 81.0
5	81.0 (C)				
6	77.1 (CH_2)	H6a: 3.81 (1H, d, 9.5)	3.65	3.65	54.1, 81.0, 85.3
		H6b: 3.65 (1H, d, 9.5)	3.81	3.81	81.0(w)
8	85.3 (C)				
9	54.1 (CH)	2.19 (1H, d, 12.0)	3.68	1.63, 3.37, 3.68	15.7, 76.9, 78.7, 85.3
10	78.7 (CH)	3.37 (1H, m)	2.09	2.09, 2.19, 2.39	15.7, 85.3, 121.9
11	30.4 (CH_2)	H11a: 2.09 (1H, m)	2.39, 3.37, 5.29	1.29, 2.39, 3.37, 5.29	78.7, 121.9, 133.3
		H11b: 2.39 (1H, m)	2.09, 5.29	1.64, 2.09, 3.37, 5.29	121.9
12	121.9 (CH)	5.29 (1H, m)	1.64, 1.71, 2.09, 2.39	1.71, 2.09, 2.39	18.1, 26.1
13	133.3 (C)				
14	15.7 (CH_3)	1.29 (3H, s)		2.09, 3.68	54.1, 85.3, 78.7
15	18.1 (CH_3)	1.64 (3H, s)	5.29	2.39	26.1, 121.9, 133.3
16	26.1 (CH_3)	1.71 (3H, s)	5.29	5.29	18.1, 121.9, 133.3
17	57.1 (CH_3)	3.39 (3H, s)		5.67	76.9
1'	166.3 (C)				
2'	123.1 (CH)	5.98 (1H, d, 15.5)	7.30	6.47, 7.30	134.1, 166.3
3'	144.0 (CH)	7.30 (1H, dd, 15.5, 11.5)	5.98, 6.47	5.98, 6.64	166.3
4'	134.1 (CH)	6.47 (1H, m)	6.64, 7.30	5.98, 6.63	139.3
5'	140.0 (CH)	6.64 (1H, m)	6.47	6.49, 7.30	133.6, 144.0
6'	139.3 (CH)	6.63 (1H, m)	6.49	6.47, 7.36	134.1, 140.0, 145.4
7'	133.6 (CH)	6.49 (1H, m)	6.63, 7.36	5.95, 6.64	140.0
8'	145.4 (CH)	7.36 (1H, dd, 15.5, 11.0)	5.95, 6.49	5.95, 6.63	170.7
9'	122.3 (CH)	5.95 (1H, d, 17.0)	7.36	6.49, 7.36	133.6, 170.7
10'	170.7 (C)				

[a] ^{1}H NMR spectra recorded at 500 MHz in $CDCl_3$, ^{13}C NMR spectra recorded at 125 MHz in $CDCl_3$.
[b] ^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by number of protons, multiplicity and coupling constant (*J*/Hz).
[c] primary, secondary, tertiary and quaternary carbons, assigned by APT.
[d] long range ^{1}H-^{13}C correlation from H to C observed in the CIGAR experiment.

Chapter 7

EXPERIMENTAL

7.1 General Methods

7.1.1 Maintenance of fungal stock cultures

Stock cultures were maintained on potato dextrose agar (PDA) slants at 4 ℃ and subcultures were taken every two months. Frozen vegetative mycelia (FVM) were prepared by aseptically transferring a portion of the slant grown on PDA plates and incubating at 25 ℃ for 3 – 4 days in the dark; agar discs (4 mm in diam.) were then cut from the growing margin of colonies and frozen in 10% glycerol in distilled water at -80 ℃ until used.

7.1.2 Culture media

CMA (corn meal agar): CMA (Difco) 17.0 g, distilled water 1000 mL.

CSA (Czapek solution agar): CSA (Difco) 49.0 g, distilled water 1000 mL.

CYA (Czapek yeast extract agar): K_2HPO_4 1 g, yeast extract (GibcoBRL) 5 g, Czapek concentrate (consisting of $NaNO_3$ 30.0 g, KCl 5.0 g, $MgSO_4$. $7H_2O$ 5.0 g, $FeSO_4$. $7H_2O$ 0.1 g and distilled water 100 mL) 10 mL, trace metal solution ($CuSO_4$. $5H_2O$ 0.5 g, $ZnSO_4$.$7H_2O$ 1 g, distilled water 100 ml) 1 mL, Sucrose 30 g, agar (GibcoBRL) 15 g, distilled water 1000 mL.

G25N (25% glycerol nitrate agar): K_2HPO_4 0.75 g, yeast extract (GibcoBRL) 3.7 g, glycerol (AR) 250 g, Czapek concentrate (consisting of $NaNO_3$ 30.0 g, KCl 5.0 g,

$MgSO_4$. $7H_2O$ 5.0 g, $FeSO_4$. $7H_2O$ 0.1 g and distilled water 100 mL) 7.5 mL, agar (GibcoBRL) 12 g, distilled water 750 mL.

MEA (malt extract agar): MEA (GibcoBRL) 50.0 g, distilled water 1000 mL.

PDA (potato dextrose agar): PDA (GibcoBRL) 39.0 g, distilled water 1000 mL.

SDA (Sabouraud dextrose agar): SDA (GibcoBRL) 65.0 g, distilled water 1000 mL.

S-PDA (seawater potato dextrose agar): PDA (GibcoBRL) 39.0 g, fresh filtered seawater (Sumner, Christchurch, New Zealand) 1000 mL.

YEA (yeast extract agar): YEA (Oxoid) 23.0 g, distilled water 1000 mL.

½ MEB (half strength malt extract broth): malt extract (Oxoid) 8.5 g, meat peptone (GibcoBRL) 1.5 g, distilled water 1000 mL.

½ PDB (half strength potato dextrose broth): PDB (Difco) 12.0 g, distilled water 1000 mL.

½ S-MEB (half strength seawater malt extract broth): malt extract (Oxoid) 8.5 g, meat peptone (GibcoBRL) 1.5 g, fresh filtered seawater (Sumner, Christchurch, New Zealand) 1000 mL.

½ S-PDB (half strength seawater potato dextrose broth): PDB (Difco) 12.0 g, fresh filtered seawater (Sumner, Christchurch, New Zealand) 1000 mL.

½ YEB (half strength yeast extract broth): yeast extract (GibcoBRL) 3.0 g, Bacto peptone 5.0 g, glucose D (+) (AR, BDH) 10.0 g, distilled water 1000 mL.

7.1.3 Biological assays[246]

Cytotoxicity

P388 murine leukaemia cell line (ATCC CCL 46, P388D1) was used. A 2-fold dilution series of the sample of interest was incubated for 72 hours with the P388 cells. The growth indicator was the yellow dye MTT tetrazolium which is reduced to purple MTT formazan by healthy cells. The result is expressed as an IC_{50} (ng/mL) which means the concentration of a sample required to reduce the P388 cell growth by

50% (comparative to control cells). The assay was carried out in 96-well microtitre plate using mitomycin C as a standard cytotoxin. Media, cell and solvent controls were included with each assay run.

Antimicrobial assay

Antimicrobial activity was detected using a zone of inhibition assay. The bacteria tested against were *Bacillus subtilis* (ATCC 19659), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). The fungi tested against were *Candida albicans* (ATCC 14053), *Trichophyton mentagrophytes* (ATCC 28185) and *Cladosporium resinae* (PAMS Dept., University of Canterbury). Each bacterium or fungus (at a known concentration) was mixed with Mueller Hinton or potato dextrose agar and poured into Petri dishes to produce a 'lawn' of bacterium/fungus. Samples of interest were then pipetted onto 6 millimetre diameter filter paper disks. The solvent was evaporated before the disks were placed onto the above prepared seeded agar. Antibiotic controls were run with each batch: Gentamycin (10 μg/disk) was used for *E.coli* and *P.aeruginosa*; Chloramphenicol (30 μg/disk) was used for *B. subtilis*; Nystatin (100 units/disk) was used for *C.albicans*, *C. resinae* and *T. mentagrophytes*. Suitable solvent controls were also used in every assay run. The result was measured in millimetres as the radius of inhibition (mass/disk).

Antiviral assay

Antiviral activity was detected using two viruses: *Herpes simplex virus* type 1 (Strain F, ATCC VR 733) and *Polio virus* type 1 (Pfiser vaccine strain). The former is a non-enveloped virus with a single standard RNA genome and a site of replication in the host cytoplasm while the latter is an enveloped virus with a double stranded DNA genome and a site of replication in the host nucleus. BSC-1 cells (African Green Monkey kidney cell line, ATCC CCL 26) were used as the host for these viruses. Samples of interest are pipetted onto 6 millimetre diameter filter paper disks and their solvents evaporated. These are then placed directly onto infected BSC-1 cells and incubated for 24 hours at 37 °C in an atmosphere containing 1.5% CO_2. The host cells were then examined with an inverted microscope for the size of antiviral (*i.e.* viral inhibition) and or cytotoxic zones, and the type of cytotoxicity.

Antiviral or cytotoxic (CYT) results:

ND no discernible antiviral or cytotoxic effects

+- minor effects located under the disc

+ antiviral or cytotoxic zone 1-2 mm excess radius from the disc (25% zone)

2+ antiviral or cytotoxic zone 2-4 mm excess radius from the disc (50% zone)

3+ antiviral or cytotoxic zone 4-6 mm excess radius from the disc (75% zone)

4+ antiviral or cytotoxic zone over the whole well (100% zone)

Cytotoxicity types (TYP): A visual interpretation of the cells change when compared to control cells: TYP1: defined nuclei; TYP2: stringy; TYP3: scruffy; TYP4: rounded; TYP5: clumped; TYP6: blown up; TYP7: granular.

7.1.4 High performance liquid chromatography (HPLC)

Analytical high performance liquid chromatography (HPLC) was carried out on a Shimadzu LC-10AD VP liquid chromatograph, equipped with a SPD-M10A VP photodiode array detector. Columns were kept at a constant temperature of 40°C with a CTO-M10AD VP oven. Reverse phase HPLC was performed on a Phenomenex Prodigy C_{18} 5-ODS column (3 μm, 250 x 4.6 mm). All samples were filtered through a syringe filter (0.45 μm) prior to injection.

Conditions for a standard reverse phase (C_{18} column) HPLC:

Mobile phase solvents: CH_3CN (ACN, HPLC grade)

H_2O (purified with a Milli-Q deionising system)

Flow rate: 1 mL/min

Solvent system: started with 10% ACN/H_2O and maintained isocratic for 2 min; a linear gradient to 75 % ACN/H_2O in 12 min; isocratic at 75 % ACN/H_2O for 10 min; a linear gradient to 100 % ACN in 2 min; isocratic at 100 % ACN for 4 min.

Semi-preparative HPLC was performed on a Shimadzu LC-4A with a Shimadzu SPD-2A S single wavelength UV detector. Solvents used were degassed with helium. Reverse phase HPLC was carried out on an Alltech C_{18} column (10 μm, 250 x 10 mm) with variable concentrations of CH_3CN (HPLC grade) in H_2O (Milli-Q).

7.1.5 Column chromatography

Column chromatography was performed with glass columns of certain dimensions. Reverse phase column chromatography was performed with J.T.Baker C_{18} (40 μm prep LC packing). Normal phase column chromatography was carried out with J.T.Baker DIOL (40 μm prep LC packing). Gel permeation column chromatography was performed on Sephadex LH-20 (Pharmacia Biotech AB). Samples were applied onto the column in two ways: Extracts were dissolved in a small amount of the initial mobile phase and applied to the top of the column bed (LH-20 and some Diol column chromatography), or, extracts were dissolved in an appropriate solvent and C_{18} or Diol (for C_{18} or Diol column chromatography, respectively) was added. The solvent was then removed under reduced pressure using a rotary evaporator and the dry power was transferred to the top of the column bed. All solvents used for chromatography were of commercial grade and distilled once, except for MeOH which was distilled twice. Reverse phase C_{18} and normal phase DIOL columns were run under pressure (0.5 kPa) with oxygen-free N_2 gas.

7.1.6 Thin layer chromatography

Thin layer chromatography (TLC) was performed on precoated Merck silica gel 60 F_{254} (0.2 mm) aluminium sheets and Merck DIOL F_{254} (0.2 mm) glass-backed plates. TLC plates were initially viewed under a UV lamp and subsequently sprayed with 10% phosphomolybdic acid in ethanol followed by heating under an IR lamp.

7.1.7 Nuclear magnetic resonance (NMR)

NMR spectra were recorded at 23°C on a Varian UNITY INOVA-500 spectrometer, operating at 500 MHz and 125 MHz for ^{1}H and ^{13}C, respectively. Other experiments described in this thesis, including, COSY, 2D-TOCSY, 2D-NOESY, HSQC and CIGAR were all recorded on the INOVA 500 spectrometer at 500 MHz. Tetramethylsilane (TMS) was used as an internal reference. The spectrometer was fitted with a pulsed field gradient MLD driver with a 5 mm Indirect Detection Probe.

Chemical shifts in this thesis are described in parts per million (ppm), on the δ scale, and referenced to the appropriate solvent peaks: $CDCl_3$ referenced to $\mathbf{C}\mathbf{H}Cl_3$ at δ_H

7.26 ppm (^{1}H) and **C**HCl$_3$ at δ_C 77.0 ppm (^{13}C); CD$_3$OD referenced to C**H**D$_2$OD at δ_H 3.30 ppm (^{1}H) and **C**HD$_2$OD at δ_C 49.3 ppm (^{13}C); Acetone-d_6 referenced to CD$_3$(C**H**D$_2$)CO at δ_H 2.17 ppm (^{1}H) and (**C**D$_3$)$_2$**C**O at δ_C 29.2 ppm and 204.1 ppm (^{13}C), respectively.

^{1}H and ^{13}C NMR spectra were recorded with acquisition time (AT) of 1.892 s and 1.3 s, respectively. 2D NOESY experiments were run using an AT of 0.150 s and a mixing time of 0.40 s. COSY and 2D TOCSY experiments were all recorded with an AT of 0.150 s and a D1 of 1.0 s. HSQC experiments were recorded with an AT of 0.140 s, a D1 of 1.0 s and $^1J_{CH}$ = 140 Hz. CIGAR experiments were run with an AT of 0.250 s, a D1 of 1.0 s and $^nJ_{CH}$ = 8.0 Hz. The spectral windows for each experiment varied according to the sample being examined.

7.1.8 Mass spectrometry

Electron impact (EI) mass spectra were obtained on a Kratos MS80RFA mass spectrometer, operating with a 4 kV accelerating potential, 70 eV and a source temperature of 250°C.

Electrospray Ionisation (ES) mass spectra were recorded on a Micromass LCT spectrometer using a probe voltage of 3200 V, an operating temperature of 150°C and a source temperature of 80°C. Samples were protonated with formic acid for positive ion mode or deprotonated with diethylamine for negative mode.

7.1.9 UV-Vis and IR spectroscopy

Ultraviolet-visible (UV-vis) spectra were recorded on a GBC UV/VIS 920 spectrometer.

Infrared (IR) spectra were recorded on a Shimadzu FTIR-8201 PC spectrometer. Spectra were obtained in a solution of chloroform or on a KBr disc.

7.1.10 Optical rotation

Optical rotations were measured with a Perkin Elmer 341 polarimeter at 20°C with a wavelength of 589 nm.

7.1.11 Solvents

Commercial grade EtOAc, DCM and petroleum ether were distilled once. Commercial grade MeOH was distilled twice and H_2O was treated by reverse osmosis and subsequently filtered through a 0.45 μm Millipore filter prior to use.

7.2 Work Described in Chapter Two

7.2.1 Growth of isolates on solid media

Six agar media were used: PDA, S-PDA, MEA, YEA, SDA and CSA.

The study on growth rate was conducted in 85 mm Petri dishes containing 20 mL of test media. All the media for a single experiment of a series of replicated experiments were made at one time and permitted to "dry" in the plates for three days before use. Inoculum was prepared by cutting agar discs, 4 mm in diam., from the perimeter of a 7-day colony grown at 25 ℃ on PDA. The inoculum discs were inverted on to the centre of Petri dishes and the dishes were sealed with parafilm to prevent drying. Then the dishes were placed in sealed plastic bags and incubated at 25 ℃ in the dark. The diameters of the 24 hour colonies were measured and used as a base to calculate the rate of growth over the following few days until the colony reached the edge of the plate. Two colony diameters, each at right angles to the other, were measured and the average of these was recorded as the colony diameter. For each medium, 5 replicated experiments were carried out.

7.2.2 Growth of isolates in liquid media

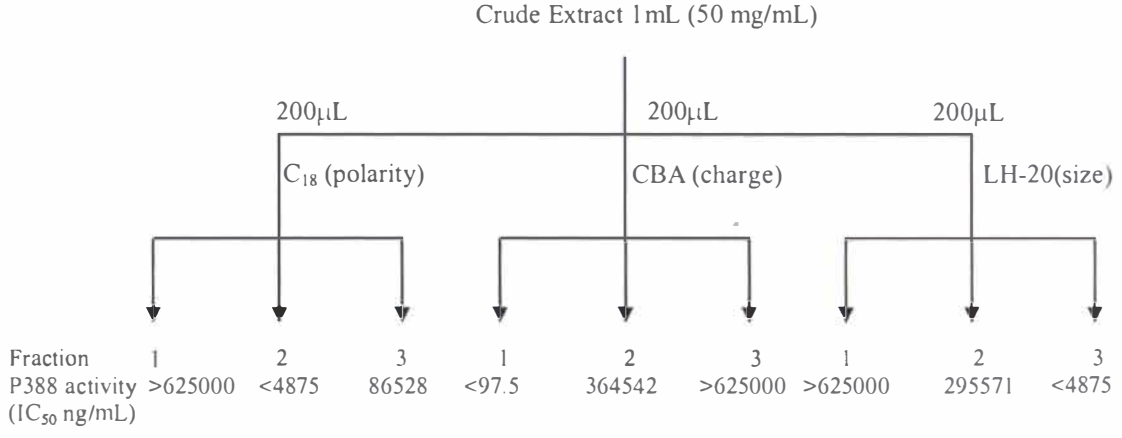
Media: ½ PDB, ½ MEB, ½ YEB, ½ S-PDB (for isolates A145 and A151), ½ S-MEB (for isolates A34, A116, A127, A128 and A266). S- means seawater.

The medium was dispensed at 125 mL per 250-mL Erlenmeyer flask, with 2 replicates for each designed condition. The flasks were closed with cotton plugs and autoclaved, then inoculated with 4 mycelial discs (8mm in diam.) cut from the growing margin of a 7-day colony at 25 °C on PDA. They were incubated at 26 °C for 28 days under shake (180 rpm) or static conditions.

The two replicated cultures were combined and homogenised with an Ultra-turrax® (Janke & Kunkle). They were then filtered through Celite 545 under suction to separate mycelium and filtrate. Both parts were extracted three times with 50 mL of EtOAc. The EtOAc solutions from the filtrate and mycelium were combined, dried over $MgSO_4$ and concentrated *in vacuo* to afford an extract. The extract was made up to 10 mg/mL in methanol for bioassay.

7.2.3 Chemical screening (1) - solid phase extraction

The extract was dissolved in methanol to make up a standard solution at 50 mg/mL. If the extract tested was less than 50 mg, then 800 μL MeOH was added so that 4 x 200 μL aliquots for screening could be obtained. 3 mL C_{18} (J.T.Baker, 40 μm) and CBA (J.T.Baker, 40 μm) cartridges and 6 mL LH-20 cartridge (contain 1.5 g LH-20) were chosen for initial screening. The solvent systems were as follows: C_{18} cartridge: 1:1 methanol/H_2O, methanol, 1:1 CH_2Cl_2/methanol. CBA cartridge: 3:2 methanol/aqueous 0.05 M ammonium acetate, 3:2 methanol/2% aqueous ammonia, 4:1 methanol/aqueous 0.05% TFA. LH-20 cartridge: straight MeOH. Three fractions from each of C_{18} and CBA cartridges and three or four fractions from the LH-20 cartridge were taken. All fractions were dried and the residues were dissolved in 200 μL solvent and submitted for P388 assay. The procedure is outlined below with the extract from fungus N60 (*Chaetomium* sp.) as an example.



In this example, the bioactive component(s) were of medium polarity (fraction 2 off C_{18}), non-ionic (eluted off CBA in fraction 1) with molecules weight smaller than 750 Daltons (retained on LH-20, fraction 3).

7.2.4 Chemical screening (2) – HPLC microtitre plate collection

The most active fraction from the LH-20 cartridge in chemical screening (1) was subjected to analytical HPLC (C_{18} column, standard conditions) and eluates were collected into a 96 well microtitre plate (Nunclon™, made in Denmark, Nalge Nunc International). For each sample, a total of 89 fractions was collected at 15 sec. intervals (approximately 250 μL/well at a flow rate 1 mL/min) and 2 min 5 sec delay. This microtitre plate was a master plate and two daughter plates were generated from it by transferring 50 μL and 5 μL aliquots respectively from each well of the master plate. The daughter plates were dried in a centrifugal evaporator and then assayed for P388 activity. Once the wells containing the bioactivity were located from the daughter plates, ESMS of the corresponding active wells in the master plate were carried out. Finally, a search on Antibase[75] with the information of UV, MS and fungus source was performed. Dereplication and the identification of the active compounds was attempted.

7.3 Work Described in Chapter Three

7.3.1 Culturing and extraction of Chaetomium sp. (A34)

The fungus strain CANU A34, collected from saline mud in western Australia, was identified as a *Chaetomium* species and deposited in the Department of Plant and Microbial Sciences, University of Canterbury, Christchurch, New Zealand (Code: CANU A34). The fungal isolate was grown on PDA Petri dishes for 7 days at 25 °C in the dark. Then mycelial disks (8 mm in diameter) were cut from the growing margin of the colony and inoculated into half strength malt extract seawater broth. A total of 11 L of broth was inoculated with 32 mycelial disks per litre of broth. The broth was incubated at 26 °C in the dark for 30 days on a rotary shaker at 180 rpm. The culture was then filtered through Celite 545 under suction to separate to a broth supernatant and a mycelial cake. The mycelial cake was washed with distilled water three times and disrupted with an Ultra-turrax®. Then the mycelium and filtrate were extracted separately with EtOAc (1 L x 3 times for mycelium, 4 L x 3 times for filtrate). The EtOAc fractions were kept overnight at 4 °C to allow the water to

separate. Then the water phase was discarded and the EtOAc phase dried with $MgSO_4$. The EtOAc extracts were evaporated under reduced pressure to yield 284.2 mg of pale yellow extract from the filtrate and 2242.9 mg of yellowish brown extract from the mycelium. The two extracts were assayed against the P388 murine leukaemia cell line and both showed significant activity (IC_{50} 234 ng/mL for filtrate extract and 25 ng/mL for mycelial extract). Since the HPLC profiles of the two extracts were similar and mycelial extract showed stronger activity, the latter was selected for chromatography.

7.3.2 Chromatography of Chaetomium sp. (A34) extracts

The mycelial extract (2000.0 mg) of *Chaetomium* sp. (A34) was separated on a reverse phase (C_{18}, 50 g) column. The column was eluted using a stepped gradient from 10% MeOH/H_2O to MeOH, increasing the concentration of MeOH in 20% steps from 10% MeOH/H_2O to 50% MeOH/H_2O and in 10% steps from 50% MeOH/H_2O to 100% MeOH every 100 mL of solvent (200 mL for 100% MeOH). Then the column was eluted with 20% CH_2Cl_2/MeOH (200 mL), 50% CH_2Cl_2/MeOH (100 mL), 100% CH_2Cl_2 (200 mL) and finally washed out with 100% MeOH (200 mL). Fifteen fractions were collected and assayed for activity against the P388 murine leukaemia cell line. Fractions A34-1-5 to A34-1-11 were active (IC_{50} <97.5 – 5199 ng/mL). The activity concentrated in the fractions A34-1-6 to 9 and decreased gradually in adjacent fractions. Checking with HPLC (C_{18} column, standard condition), fractions A34-1-6, 7, 8, 9 and 10 showed nearly the same HPLC profile: all having a major peak at RT 15.00 minutes with several minor peaks differing only in ratios. Fractions A34-1-5 and A34-1-11 showed different HPLC profiles with a very small peak at RT 15.00 minutes. Therefore the peak at RT 15.00 minutes represented the major activity observed. The ^1H NMR spectra also revealed that fractions A34-1-6, 7, 8, 9 and 10 contained the same major compound. This compound was in higher concentrations in the fractions A34-1-6 and 7 while in relatively lower concentrations in fractions A34-1-8, 9 and 10. A34-1-11 was predominantly fatty acids and A34-1-5 was a complex mixture.

Fractions A34-1-6 (166 mg) and A34-1-7 (200 mg) were combined and

chromatographed using a normal phase (Diol, 15 g) column. The column was eluted accordingly with 5%, 10%, 20%, 40%, 60% and 80% EtOAc/CH_2Cl_2 (20 mL for each concentration), 100% EtOAc (20 mL), 5% MeOH/EtOAc (15 mL), 10% MeOH/EtOAc (15 mL) and finally washed out with 100% MeOH (35 mL). Thirteen fractions were collected and combination of the fractions was based on the analysis of TLC. Although all the fractions showed activity against the P388 cell line, fractions A34-D1 and D2 were the most active and the activity decreased gradually in the following fractions. The HPLC profiles of fractions A34-D2 to D6 were very close, all showing a major peak at RT 15.00 minutes and several minor peaks varying in different fractions. Fractions A34-D7 to D13 only had a minor peak at 15.00 minutes. It was then confirmed that the peak of RT 15.00 minutes in the HPLC (C_{18} column, standard conditions) profiles was responsible for the P388 activity and that the compound represented by this peak had poor solubility in the solvent used. Because the active compound had the highest concentration and the largest mass in A34-D2, this fraction was used for further purification.

A34-D2 (65.0 mg) was further purified using a semi-preparative HPLC (C_{18} column, reverse phase), eluted with 40% ACN/H_2O isocratically. Although six peaks were collected, only fraction A34-D2-2, collected from a major peak of the HPLC profile was a pure compound (47.9 mg). A34-D2-2 showed very significant activity against the P388 cell line with IC_{50} 11.2 ng/mL. Fractions A34-D2-1 and A34-D2-6 were complex mixtures with a small amount of A34-D2-2. Fractions A34-D2-3, 4 and 5 were mixtures in which A34-D2-2 was still a major compound together with two or three related compounds. To obtain these minor compounds, A34-D2-3 (1.7 mg) was further chromatographed with a long, thin LH-20 column (2.5 g), washed with straight MeOH. No pure compounds were obtained. A34-D2-4 (3.0 mg) was further purified with another semi-preparative HPLC (C_{18} column, reverse phase), eluted with 48% ACN/H_2O isocratically. Again, no pure related compounds were achieved.

To obtain compounds related to A34-D2-2, fraction A34-1-9 was selected for further chromatography because this fraction showed a relatively higher content of related compounds in the HPLC profile. A34-1-9 (306 mg) was applied onto normal phase (Diol, 15 g) column, eluted by a gradient solvent system from 100% CH_2Cl_2 to EtOAc

and then to MeOH. Sixteen fractions were collected and the related compounds were found in the fractions A34-9-2 and A34-9-3 by HPLC and ^{1}H NMR spectra. Fraction A34-9-3 (31.3 mg) was further chromatographed with another Diol column (15 g, normal phase), using a gradient solvent system from 50% hexane/CH_2Cl_2 to CH_2Cl_2 and then to EtOAc. Fifteen fractions were collected and related compounds were located in the fractions A34-9-3-6 and A34-9-3-7. These two fractions were purified separately by semi-preparative HPLC (C_{18} column, reverse phase), eluted with 45% ACN/H_2O isocratically. Possibly due to the same reason mentioned above, no pure related compounds were obtained.

Fraction A34-9-2 (111.1 mg) was further purified on a second Diol column (16 g, normal phase), eluted with a solvent system from 100% hexane to CH_2Cl_2 and then to EtOAc. Fourteen fractions were collected and the related compounds were located in fractions A34-9-2-7 and A34-9-2-8. Since these two fractions showed nearly the same HPLC profiles as that of A34-9-3-6 and A34-9-3-7, no further work was carried out.

7.3.3 Taxonomy of Chaetomium sp. (N60)

Media used in taxonomy: Malt extract agar (MEA) and 25 % glycerol nitrate agar (G25N) (see section **7.1.2**).

Ten replicated experiments were carried out for each condition.

7.3.4 Culturing and extraction of Chaetomium globosum (N60)

A strain of *Chaetomium globosum* (CANU N60) was collected from saline sand in New Zealand and deposited in the culture collection of the Department of Plant and Microbial Sciences, University of Canterbury, Christchurch, New Zealand. The isolate was grown on PDA solid medium for 5 days at 25 °C in the dark. Mycelial disks (8 mm in diameter) were cut from the outer regions of the colony and used for incubating ½ MEB (see section **7.1.2**) cultures. Two litres of such broth were inoculated (32 mycelial disks per litre of broth) and incubated at 26 °C in the dark for four weeks on a rotary shaker at 180 rpm. The culture was then filtered through Celite 545 under suction to separate mycelium and filtrate. The mycelium was washed with distilled water three times and disrupted with an Ultra-turrax®. Then the mycelium

and filtrate were extracted separately with EtOAc (200 mL x 4 times for mycelium, 2 L x 4 times for filtrate). The extracts were dried with $MgSO_4$ and evaporated under reduced pressure to yield 234 mg of yellowish brown extract from the filtrate and 294 mg of brownish red extract from the mycelium. The two extracts were assayed against the P388 murine leukaemia cell line and both showed significant activity (IC_{50} 1942 ng/mL for filtrate extract and 2325 ng/mL for mycelial extract). Because the HPLC profiles of the two extracts were identical, they were combined before further purification.

7.3.5 Chromatography of Chaetomium globosum (N60) extract

The crude extract (480 mg) was chromatographed on a reverse phase (C_{18}, 50 g) column. The column was eluted using a stepped gradient from 10% MeOH/H_2O to MeOH, increasing the concentration of MeOH in 20% steps from 10% MeOH/H_2O to 50% MeOH/H_2O and in 10% steps from 50% MeOH/H_2O to 90% MeOH/ H_2O every 100 mL of solvent. Then the column was eluted with 100% MeOH (200 mL), 20% CH_2Cl_2/MeOH (100 mL), 50% CH_2Cl_2/MeOH (100 mL), 100% CH_2Cl_2 (200 mL) and finally washed out with 100% MeOH with 0.05% TFA (200 mL). Fourteen fractions were collected and combination of the fractions was based on HPLC analysis. On the assay against the P388 murine leukaemia cell line, fractions N60 1.5, 1.6, 1.7 and 1.8 (eluted with 70% MeOH/H_2O, 70% MeOH/H_2O, 80% MeOH/H_2O and 90% MeOH/H_2O respectively) showed significant activities (IC_{50} 8469, 3660, 3660 and 3886 ng/mL, respectively). Fraction N60 1.9 (eluted with 100% MeOH) exhibited weak P388 activity (IC_{50} 12134 ng/mL).

7.3.5.1 Separation of fraction N60 1.6

Fraction N60 1.6 (116.5 mg) was applied on a normal phase (Diol, 16 g) column, eluted with a stepped gradient from CH_2Cl_2 to EtOAc then to MeOH: 100% CH_2Cl_2 (30mL), 5% EtOAc/CH_2Cl_2 (20 mL), 30% EtOAc/CH_2Cl_2 (20 mL), 50% EtOAc/CH_2Cl_2 (10 mL), 80% EtOAc/CH_2Cl_2 (10 mL), 100% EtOAc (10 mL), 5% MeOH/EtOAc (20 mL), 50% MeOH/EtOAc (20 mL) and 100% MeOH (20 mL). Fifteen subfractions were collected and combination of the subfractions was based on HPLC. The UV profiles revealed that compounds of interest were collected in

subfractions N60 1.6.3 to 9. Subfractions N60 1.6.7 and 1.6.8, eluted with 50% and 80% EtOAc/CH_2Cl_2 respectively, gave a pure compound [N60 1.6.7+8, 4.9 mg]. Subfractions N60 1.6.3 and N60 1.6.4, eluted with 5% EtOAc/CH_2Cl_2, contained a major compound of crude N60 1.6. Because subfraction N60 1.6.3 contained less impurity than N60 1.6.4, the former was selected for further purification. Subfraction N60 1.6.3 was applied on another Diol column (12 g) and eluted with 70% CH_2Cl_2/hexanes isocratically. Thirty-four fractions were collected. The expected pure compound (N60 1.6.3.10-19, 4.9 mg, a major compound of N60 1.6) was obtained in the fractions N60 1.6.3.10 to N60 1.6.3.19.

7.3.5.2 Separation of fraction N60 1.7

Fraction N60 1.7 (78.5 mg) was further chromatographed by a Diol (16 g) column, eluted with gradient solvent system of CH_2Cl_2/EtOAc/MeOH: 100% CH_2Cl_2 (30 mL), 5%, 10%, 20%, 50%, 80% EtOAc/CH_2Cl_2 (20 mL each), 100% EtOAc (10 mL), 50% MeOH/EtOAc (20 mL), 80% MeOH/EtOAc (20 mL) and 100% MeOH (30 mL). A total of seventeen subfractions was collected and combination of the subfractions was based on HPLC analysis. Subfractions N60 1.7.4 to N60 1.7.10 contained compounds of interest. N60 1.7.4 and N60 1.7.5 was a pure compound (14.4 mg) which represented a major peak on the HPLC profile of the crude extract. Subfractions N60 1.7.6 to 10 were mixtures containing the same compound of interest. Among these fractions, N60 1.7.8 was the most promising one for obtaining the expected compound. Subfraction N60 1.7.8 (2.6 mg) was further purified with a LH-20 (2 g) column, eluted with straight MeOH. The expected pure compound was obtained in fraction N60 178.8 (1.5 mg).

The UV profiles and ^{1}H NMR spectra revealed that fractions N60 1.7.2 and N60 1.7.3 contained compounds totally different from the major compounds in the crude extract. Subfractions N60 1.7.3 was a pure compound while N60 1.7.2 consisted of equal amounts of compound N60 1.7.3 and a compound related to it, namely N60 1.7.2A. Because no P388 activity was detected in these two fractions and the ^{1}H NMR signals of compound N60 1.7.2A could be distinguished from that of N60 1.7.3, no further purification was attempted for fraction N60 1.7.2.

7.3.5.3 Separation of fraction N60 1.8

Fraction N60 1.8 (15.2 mg) was further chromatographed on a Diol (normal phase, 12 g) column. The column was eluted with a gradient solvent system consisting of hexanes-EtOAc-MeOH: 100% hexanes (30 mL), 10% EtOAc/hexanes (30 mL), 20% EtOAc/hexanes (60 mL), 30% EtOAc/hexanes (30 mL), 40% EtOAc/hexanes (30 mL), 60% EtOAc/hexanes (30 mL), 80% EtOAc/hexanes (30 mL), 100% EtOAc (50 mL), 20% MeOH/EtOAc (30 mL), 50% MeOH/EtOAc (30 mL), 100% MeOH (50 mL). A total of 29 subfractions was collected and combination of the subfractions was based on the analysis of ^{1}H NMR spectra. Subfractions N60 1.8.14 and 15, eluted with 100% EtOAc, yielded a pure compound (N60 1.8.14+15, 7.9 mg) structurally related to compound N60 1.7.4+5. Subfractions N60 1.8.12 and 13, eluted with 80% EtOAc/hexanes were combined. The combined subfraction (N60 1.8.12+13) was relatively pure and contained only two compounds which were structurally similar to compound N60 1.8.14+15. The ratio of the two compounds was nearly 1:1. Due to the small amount of mass (6.1 mg) and due to the fact that the ^{1}H NMR signals of the two compounds could be distinguished from each other in the spectrum, no further purification was carried out for this subfraction.

7.3.5.4 Separation of fraction N60 1.5

Fraction N60 1.5 (52.3 mg) was further purified using a normal phase (Diol, 12 g) column. The column was eluted with a gradient solvent system as the following: 10% to 100% EtOAc/hexanes (increasing EtOAc concentration in 10% steps every 30 mL of solvent from 10% to 60% EtOAc/hexanes and every 50 mL of solvent from 70% EtOAc/hexanes to 100% EtOAc), 20% MeOH/EtOAc (30 mL), 50% MeOH/EtOAc (30 mL) and 100% MeOH (100 mL). A total of 30 subfractions was collected and combination of the subfractions was based on the analysis of ^{1}H NMR spectra. Subfraction N60 1.5.10, eluted with 80% EtOAc/hexanes, gave a pure compound (N60 1.5.10, 5.0 mg) which was structurally related to the major compound N60 1.7.4+5. Subfractions N60 1.5.11, 12 and 13, eluted with 90% EtOAc/hexanes, contained a compound of interest. The three subfractions were combined (13.5 mg) and applied onto another Diol column (16 g) for further purification. The column was

eluted with a solvent system consisting of EtOAc and hexanes, increasing EtOAc concentration in 10% steps from 30% to 60% EtOAc/hexanes every 30 mL of solvent, then to 70% EtOAc/hexanes (150 mL), 80% EtOAc/hexanes (30 mL), 90% EtOAc/hexanes (30 mL) and finally to 100% EtOAc (50 mL). Nine fractions were collected. Fractions N60 1511.4 and N60 1511.5, eluted with 70% EtOAc/hexanes, yielded a pure compound (N60 1511. 4+5, 5.1 mg).

7.3.5.5 Separation of fractions N60 1.9 and N60 1.10

The ^{1}H NMR spectra of fractions N60 1.9 and N60 1.10 were identical but different from that of the major compound N60 1.7.4+5. Although fraction N60 1.9 showed mild activity while fraction N60 1.10 was inactive in the assay against the P388 cell line (IC_{50} 12134 and >12500 ng/mL, respectively), they were combined for further purification.

The combined fraction (total 22.1 mg) was chromatographed with a Diol (normal phase, 12 g) column. The column was eluted with a gradient solvent system from hexanes to EtOAc then to MeOH: 100% hexanes (50 mL), 5% EtOAc/hexanes (50 mL), 20% EtOAc/hexanes (100 mL), 30% to 70% EtOAc/hexanes (increasing EtOAc concentration in 10% steps every 50 mL of solvent), 90% EtOAc/hexanes (40 mL), 100% EtOAc (50 mL), 50% MeOH/EtOAc (50 mL), 100% MeOH (50 mL). A total of twenty-eight subfractions was collected and combination of the subfractions was based on analysis of ^{1}H NMR spectra. Subfraction N60 1910.1a, eluted with 100% hexanes to 30% EtOAc/hexanes, contained a target compound. This subfraction (6.6 mg) was further purified by another normal phase (Diol, 10 g) column, eluted with a solvent system of EtOAc/hexanes. The EtOAc concentration was increased in 5% steps from 100% hexanes to 30% EtOAc/hexanes every 40 mL of solvent, then the column was washed out with 50% EtOAc/hexanes (50 mL). Twenty-six fractions were collected. The expected pure compound (N60 1910.1a.22-25, 3.2 mg) was obtained in fractions N60 1910.1a.22, 23, 24 and 25, eluted with 30% EtOAc/hexanes.

Chaetoglobosin Q (N60 1.6.7+8) $[\alpha]_D^{20}$ -100° (c 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 220 (4.46), 282 (3.73), 291 (3.67) nm; IR ($CHCl_3$) ν_{max} 3689, 3544, 3060-3240

(br), 2360, 1695, 1685, 1602, 1188 cm^{-1}.

Chaetoglobosin R (N60 1511.4+5) $[\alpha]_D^{20}$ -100° (c 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 217 (4.81), 289 (4.05) nm; IR ($CHCl_3$) ν_{max} 3689, 3535, 3080-3240 (br), 2360, 1691, 1683, 1602, 1193 cm^{-1}.

Chaetoglobosin S (N60 178.8) Obtained as colourless powder; $[\alpha]_D^{20}$ -80° (c 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 220 (4.39), 283 (3.58), 290 (3.54) nm; IR ($CHCl_3$) ν_{max} 3691, 3481, 2360, 2341, 1697, 1685, 1604, 1463 cm^{-1}.

7.4 Work Described in Chapter Four

7.4.1 Culturing and extraction of Phoma sp. (K31)

A *Phoma* sp. (K31) was isolated from a driftwood sample in Canada and deposited in the culture collection of the Department of Plant and Microbial Sciences, University of Canterbury, Christchurch, New Zealand (CANU K31). It was grown on PDA Petri dishes for 8 days at 25 °C in the dark. Mycelial disks (8 mm in diameter) were cut from the growing edge of the colony and transferred into ½ PDB (32 mycelial disks per litre of broth). The broth was incubated at 26 °C in the dark under static conditions. Two batches were cultured. The first batch (6 L), big broth I, was cultured for four weeks and the second batch (2 L), big broth II, was cultured for eleven weeks. Each culture was filtered through Celite 545 under suction to separate to a broth supernatant and a mycelial cake. The mycelial cake was washed with distilled water three times and disrupted with an Ultra-turrax®. The filtrate and mycelium were extracted separately with EtOAc (200 mL x 3 times per litre of filtrate and 200 mL x 3 times for each mycelial cake). The EtOAc extracts were dried with $MgSO_4$ and then evaporated under reduced pressure. They were then assayed for activity against the P388 murine leukaemia cell line. The filtrate extract (867.8 mg) from big broth I showed moderate activity (IC_{50} 6248 ng/mL) while filtrate extract from big broth II and mycelial extracts from both big broth I and II showed no activity (IC_{50} >12500 ng/mL). The active extract was therefore selected for further purification.

7.4.2 Chromatography of Phoma sp. (K31) extract

The filtrate extract (867.8 mg) from big broth I (6 L) of *Phoma* sp. (K31) was chromatographed on a reverse phase (C_{18}, 50 g) column. The column was eluted using a stepped gradient from 10% MeOH/H_2O to MeOH, increasing the concentration of MeOH in 20% steps from 10% MeOH/H_2O to 50% MeOH/H_2O and in 10% steps from 50% MeOH/H_2O to 100% MeOH every 100 mL of solvent, then the column was eluted with 20% CH_2Cl_2/MeOH (100 mL), 50% CH_2Cl_2/MeOH (100 mL) and 100% CH_2Cl_2 (100 mL). Fourteen fractions were collected and combination was based on TLC (silica gel 60 F_{254}). The first three fractions (K31-1 to 3), eluted by 10-50% MeOH/H_2O, showed activity against the P388 murine leukaemia cell line. Since the P388 activities of fractions K31-1 and K31-3 were very weak (IC_{50} >10, 000 ng/mL), only fraction K31-2 (IC_{50} 8688 ng/mL) was selected for further chromatography.

Fraction K31-2 (256.3 mg) was purified using a LH-20 column (12 g), eluted with MeOH isocratically. Fourteen fractions were collected and combination was based on TLC (silica gel 60 F_{254}). Though all the fractions (2 mg/mL) were submitted for P388 assay, only very weak activities were recovered from fractions K31-2-3 and K31-2-4 (IC_{50} 18531 and 17984 ng/mL, respectively).

Fractions K31-2-3 (109.0 mg) and K31-2-4 (29.9 mg) showed the same HPLC profiles. They were combined and applied onto another LH-20 (12 g) column. The column was washed with straight MeOH and seven fractions were collected. The P388 activity was found in the fraction K31-2-(3+4)-4, though still very weak (IC_{50} 18224 ng/mL). Fraction K31-2-(3+4)-4 was renamed as K31-a due to the long code.

The fraction K31-a (97.9 mg) was further chromatographed on a normal phase (Diol, 15 g) column, eluted with a gradient solvent system composed of hexane/EtOAc (1:2, 80 mL), 100% EtOAc (80 mL) and EtOAc/MeOH (1:1, 80 mL). Sixteen fractions were collected and combination was based on TLC (Diol). The HPLC profiles and ^1H NMR spectra of these fractions indicated that K31-a-6 to a-11 was a pure compound (5.2 mg, 2,5-dihydroxybenzoic acid, see **Chapter 4, 4.3**), K31-a-4 was a mixture of which the major compound structurally related to K31-a-6 to a-11, K31-a-5 was a mixture of K31-a-4 and K31-a-6 to a-11, K31-a-2 and K31-a-3 were complex

mixtures where the major compound was the same as that of K31-a-4, K31-a-1 and K31-a-12 to a-16 showed no signals in the ^1H NMR experiment. K31-a-4 was selected for further purification.

Fraction K31-a-4 (9.2 mg) was further purified using a semi-preparative HPLC (C_{18} column, reverse phase), eluted with 5% ACN/H_2O isocratically. Three peaks were collected. The expected pure compound was obtained in the fraction K31-a-4-2 (1.3 mg, 2,5–dihydroxybenzyl alcohol, see **Chapter 4, 4.4**). Fractions K31-a-4-1 (1.1 mg) and K31-a-4-mixture (3.5 mg) were impure mixtures.

7.5 Work Described in Chapter Five

7.5.1 Culturing and extraction of Fusarium sp. (A145)

A *Fusarium* sp. (A145) was isolated from saline lake sand in western Australia and deposited in the culture collection of the Department of Plant and Microbial Sciences, University of Canterbury, Christchurch, New Zealand (Code: CANU A145). The isolate was grown on PDA Petri dishes for 7 days at 25 °C in the dark. Mycelial disks (8 mm in diameter) were cut off from the growing margin of the colony and transferred into half strength potato dextrose broth. Four litres of broth were inoculated with 32 mycelial disks per litre of broth. The broth was cultured for 5 weeks at 26 °C on a rotary shaker at 180 rpm. The culture was then filtered through Celite 545 under suction to separate to a broth supernatant and a mycelial cake. The filtrate and mycelium were extracted separately with EtOAc (1 L x 3 times for filtrate and 300 mL x 3 times for mycelium). The EtOAc fractions were kept overnight in the refrigerator to allow water to separate. The water phase was discarded and the EtOAc phase dried with $MgSO_4$. The EtOAc extracts were then evaporated under reduced pressure and yielded 243.6 mg of dark red extract from the filtrate and 674.8 mg of orange extract from the mycelium. The two extracts were assayed against the P388 cell line. The filtrate extract showed strong activity while the mycelium extract exhibited very weak activity (IC_{50} < 975 and 23167 ng/mL, respectively). Thus, the filtrate extract was selected for chromatography.

7.5.2 Isolation of compound 5.2 (beauvericin)

The filtrate extract (243.6 mg) of *Fusarium* sp. (A145) was chromatographed on a reverse phase (C_{18}, 50 g) column. The column was eluted using a stepped gradient from 10% MeOH/H_2O to MeOH, increasing the concentration of MeOH in 20% steps from 10% MeOH/H_2O to 50% MeOH/H_2O and in 10% steps from 50% MeOH/H_2O to 100% MeOH every 100 mL of solvent. The column was then eluted with 20% CH_2Cl_2/MeOH, 50% CH_2Cl_2/MeOH and 100% CH_2Cl_2 in 100 mL for each kind of solvent system and finally washed with 200 mL of 100% MeOH. Fourteen fractions were collected. Fractions WJ3.25.7, 8, 9, 10, 11 and 12, eluted by 90% MeOH/H_2O to 100% CH_2Cl_2, showed significant activity against the P388 Murine leukaemia cell line (IC_{50} =183 - 4651 ng/mL).

The ^{1}H NMR spectra showed that fractions WJ3.25.7 and 8 contained the same major compound. The HPLC profiles revealed that WJ3.25.8 was a pure compound (87.2 mg, compound 5.2, beauvericin, see section **5.5 , Chapter 5**) while WJ3.25.7 was a mixture with compound 5.2 being the major one (> 90%). Therefore, no further work was carried out for WJ3.25.7.

EI-MS for WJ3.25.8: *m/z* 783 [M^+], 692, 592, 505, 431, 392, 331, 262, 244, 170, 134, 91

7.5.3 Isolation of compound 5.1 (bikaverin)

The active fractions WJ3.25.11 and 12 were combined (60.8 mg, WJ3.25.11+12) and further chromatographed on a normal phase (Diol, 16 g) column, eluted with 100% hexanes (30 mL), 10 % CH_2Cl_2/hexanes (30 mL), 20 - 90 % CH_2Cl_2/hexanes (increasing CH_2Cl_2 concentration in 10% steps every 50 mL of solvent), 100 % CH_2Cl_2 (70 mL) and then 50% MeOH/CH_2Cl_2 (50 mL) and 100% MeOH (100 mL). A total of 37 subfractions was collected and combination was based mainly on ^{1}H NMR spectra. The active fractions – subfractions WJ3.28.13 to 32, actually contained only one compound and this compound always mixed with fatty acid. The poor solubility of this compound (compound 5.1) was suggested by its broad distribution in 10 consecutive subfractions. Subfractions WJ3.28.13 to 32 were combined and dried (20.4 mg), and then washed with hexanes (10 mL x 3) to remove fatty acid. The

residue was dried and then crystallised with chloroform to achieve red needles (7.5 mg). The red needles were compound 5.1 (bikaverin, see section **5.3 , Chapter 5**).

7.5.4 Isolation of compound 5.3 (bassiatin)

The ^{1}H NMR spectra of WJ3.25.9 and 10 indicated that they were mixtures of WJ3. 25.8 (beauvericin) and WJ3.25.11-12 (bikaverin) and therefore, no further work was conducted with these two fractions. Fraction WJ3.25.5, though inactive in the assay against the P388 cell line (IC_{50} > 12500 ng/mL), had a ^{1}H NMR spectrum that showed the major compound in this fraction might be structurally similar to compound 5.2. Therefore, further purification was carried out for this fraction. Fraction WJ3.25.5 (12.8 mg) was applied on a normal phase (Diol, 20 g) column, eluted with a gradient solvent system from hexanes to CH_2Cl_2 then to MeOH: 0% to 60% CH_2Cl_2/hexanes (increasing CH_2Cl_2 concentration in 20% steps every 20 mL of solvent), 80% CH_2Cl_2/hexanes (100 mL), 100% CH_2Cl_2 (50 mL), 10% MeOH/CH_2Cl_2 (50 mL), 50% MeOH/CH_2Cl_2 (50 mL) and 100% MeOH (100 mL). Twenty one subfractions were collected and combination was based mainly on ^{1}H NMR spectra. The metabolite closely related to compound 5.2 was collected in subfraction WJ3.31.1, eluted with 100% hexanes to 60% CH_2Cl_2/hexanes and was shown to be pure by HPLC (0.9 mg, compound 5.3, bassiatin, see section **5.4 Chapter 5**).

7.6 Work Described in Chapter Six

7.6.1 Taxonomy of Aspergillus fumigatus (A151)

Media used in taxonomy: Czapek yeast extract agar (CYA), 25 % glycerol nitrate agar (G25N) and malt extract agar (MEA) (see section **7.1.2**).

Ten replicated experiments were carried out for each condition.

7.6.2 Culturing and extraction of Aspergillus fumigatus (A151)

An *Aspergillus fumigatus* (CANU A151) was isolated from saline lake sand in western Australia and deposited in the culture collection of the Department of Plant and Microbial Sciences, University of Canterbury, Christchurch, New Zealand (Code: CANU A151). The isolate was grown on PDA plates for 7 days at 25 °C, then

mycelial disks (8 mm in diameter) were transferred into liquid medium (32 disks per litre of liquid medium) consisting of half strength potato dextrose broth (1/2 PDB) and then incubated at 26 °C on a rotary shaker at 180 rpm. Two batches were cultured. The first batch (5 L), big broth I, was cultured for four weeks and the second batch (2 L), big broth II, was cultured for eleven weeks. Each culture was filtered through Celite 545 under suction to separate to a broth supernatant and a mycelial cake. The mycelial cake was washed with distilled water three times and disrupted with an Ultra-turrax®. The mycelium and filtrate were extracted three times separately with EtOAc. The EtOAc extracts were then evaporated under reduced pressure and assayed against the P388 murine leukaemia cell line. Filtrate extract from big broth I and the mycelium extract from big broth II showed significant activity against the P388 murine leukaemia cell line (IC_{50} 2189 and 1207 ng/mL, respectively). The two active extracts were used for further purification.

7.6.3 Chromatography of mycelial extract from big broth II

The mycelial extract (227.7 mg) from big broth II was washed with hexanes three times. The residue and hexanes extract were then analysed by ^{1}H NMR. The ^{1}H NMR spectrum of the hexanes extract showed characteristic fatty acid peaks as identified previously while the spectrum of the residue showed totally different signals. The residue (118.3 mg) was applied to a Diol (normal-phase, 20 g) column. The column was eluted using a stepped solvent gradient from 100% hexanes to 80% EtOAc/ hexanes, increasing the EtOAc concentration in 20% steps every 50 mL of solvent, then 100% EtOAc (200 mL), 20% MeOH/ EtOAc (50 mL), 50% MeOH/ EtOAc (50 mL) and finally eluted with 100% MeOH (50 mL). A total of seventeen fractions was collected. Biological assessment of the fractions located P388 activity in fractions WJ3.42.1, 2, 3, 4 and 10 to 13 (IC_{50} 606 – 2795 ng/mL). By ^{1}H NMR and HPLC profile, Fraction WJ3.42.2, eluted with 40-60% EtOAc/hexanes was shown to be a pure compound which was identified as xanthocillin – X dimethyl ether (see **Chapter 6, 6.4**). Fractions WJ3.42.1, 3 and 4 were predominatly compound WJ3.42.2 with very few impurities. Fractions WJ3.42.10 to 13 gave the same ^{1}H NMR and HPLC profile to that of WJ 3.40.11 to 14. For further purification see section **7.6.4** (Fraction 3.37.6, then subfraction WJ 3.40.11 to 14).

7.6.4 Chromatography of filtrate extract from big broth I

The filtrate extract (590.9 mg) of big broth I was separated on a reverse phase (C_{18}, 50 g) column. The column was eluted using a stepped gradient from 10% MeOH/H_2O to 90% MeOH, increasing the concentration of MeOH in 20% steps from 10% MeOH/H_2O to 50% MeOH/H_2O and in 10% steps from 50% MeOH/H_2O to 90% MeOH/H_2O every 100 mL of solvent. Then the column was eluted with 100% MeOH (200 mL), 20% CH_2Cl_2/MeOH (100 mL), 50% CH_2Cl_2/MeOH (100 mL), 100% CH_2Cl_2 (200 mL) and finally washed with 100% MeOH (80 mL). Fifteen fractions were collected. The activity against the P388 cell line was located in fractions WJ3.37.2 (IC_{50} = 2880 ng/mL, 17.2 mg), WJ3.37.6 (IC_{50} = 7976 ng/mL, 107.0 mg) and WJ3.37.7 (IC_{50} = 7741 ng/mL, 62.9 mg), eluted with 30% MeOH/H_2O, 70% MeOH/H_2O and 80% MeOH/H_2O, respectively.

Fraction WJ3.37.7 (62.9 mg) was further chromatographed on a normal phase (Diol, 20 g) column, eluted with a gradient solvent system from 50% CH_2Cl_2/hexanes to 100% CH_2Cl_2 then to 100% MeOH: 50% CH_2Cl_2/hexanes (50 mL), 80% CH_2Cl_2/hexanes (50 mL), 100% CH_2Cl_2 (50 mL), 20% EtOAc/CH_2Cl_2 (50 mL), 40% EtOAc/CH_2Cl_2 (50 mL), 60% EtOAc/CH_2Cl_2 (100 mL), 80% EtOAc/CH_2Cl_2 (50 mL), 100% EtOAc (50 mL), 50% MeOH/EtOAc (50 mL) and 100% MeOH (100 mL). A total of 28 subfractions was collected and combination was based mainly on ^{1}H NMR spectra. By checking with HPLC, subfractions WJ3.47.2+3, WJ3.47.5 and WJ3.47.8+9, eluted with 20%, 20% and 40% EtOAc/CH_2Cl_2 respectively, afforded three pure compounds. WJ3.47.8+9 proved to be a new compound which was named fumagiringillin (see section **6.7 Chapter 6**). WJ3.47.2+3 (see section **6.5 Chapter 6**) and WJ3.47.5 (see section **6.6 Chapter 6**) were determined to be the known compounds - 12-α fumitremorgin C and fumagillin respectively.

Although fraction WJ3.37.7 showed activity in the P388 assay ((IC_{50} 7741 ng/mL), no such activity was detected in the subfractions (WJ3.47.x) including the three pure compounds after Diol column chromatography. The loss of activity might be due to decomposition of active component (s) or that the activity of fraction WJ3.37.7 was a synergistic effect of inactive components in subfractions WJ3.47.x.

Fraction WJ3.37.6 (107.0 mg) was further purified using a Diol (normal phase, 20 g) column, eluted with a gradient solvent system of hexanes - CH_2Cl_2 – EtOAc – MeOH. Forty subfractions were collected. Subfraction WJ3.40.11 to 14, eluted with 40-60% EtOAc/ CH_2Cl_2, showed activity against the P388 murine leukaemia cell line (IC_{50} = 2795 ng/mL). The solubility of WJ3.40.11 to 14 was very poor in all solvents available. Therefore, crystallisation was used for purification and white crystals obtained in acetone. The poor solubility meant the ^{13}C NMR and CIGAR data were very limited and no structural identification was achieved.

Fraction WJ3.37.2 (17.2 mg) was applied onto a normal phase (Diol, 20 g) column and eluted using a gradient solvent system consisting of hexanes, CH_2Cl_2, EtOAc and MeOH. Thirty three subfractions were collected and combination was made by analysis of ^1H NMR spectra. Subfractions WJ3.54.11 and WJ3.54.12 to 16, eluted with 60% and 80% EtOAc/DCM respectively, showed activity against the P388 cell line (IC_{50} = 3552 and 3552 ng/mL, respectively). The ^1H NMR spectra of the active fractions revealed that the major components in these fractions were simple benzenoid compounds structurally related to those isolated previously from the extract of *Phoma sp* (K31) (see **Chapter 4**). Due to the relatively simple chemical structure and the low yield at this stage of purification, the compounds responsible for the activity in these fractions were not pursued further.

WJ3.47.8+9 Obtained as pale yellow powder; $[\alpha]^{20}_D$ –12.5° (c 1.0, MeOH); UV (MeOH) λ_{max} (log ε) 317 (sh, 2.18), 332 (2.29), 348 (2.23) nm; IR ($CHCl_3$) ν_{max} 3687, 3398 (br), 2972, 1706, 1627, 1236, 1051, 1041 cm^{-1}.

REFERENCES

(1) Hawksworth, D. L. *Mycol. Res.* **1991**, *95*, 641-655.

(2) Demain, A. L.; Fang, A. *Adv. Biochem. Eng. Biotechnol.* **2000**, *69*, 1-39.

(3) Turner, W. B.; Aldridge, D. C. *Fungal Metabolites II;* Academic Press INC. (London) LTD: London, 1983.

(4) Pearce, C. *Biologically Active Fungal Metabolites.* In *Advances in Applied Microbiology;* Neidleman, S. L., Laskin, A. I., Eds.; Academic: San Diego, 1997; Vol. 44, pp 1-80.

(5) Venkata Dasu, V.; Panda, T. *Biopro. Eng.* **1999**, *21*, 489-495.

(6) Davies, R. R. *Antifungal Chemother.* **1980**, 149-182.

(7) Verbist, L. *J. Antimicrob. Chemother.* **1990**, *25*, 1-5.

(8) Weil, C. *Med. Res. Rev.* **1984**, *4*, 221-265.

(9) Sievers, T. M.; Rossi, S. J.; Ghobrial, R. M.; Arriola, E.; Nishimura, P.; Kawano, M.; Holt, C. D. *Pharmacotherapy* **1997**, *17*, 1178-1197.

(10) Alberts, A. W.; Chen, J.; Kuron, G.; Hunt, V.; Huff, J.; Hoffman, C.; Rothrock, J.; Lopez, M.; Joshua, H.; *et al. Proc. Natl. Acad. Sci. U. S. A.* **1980**, *77*, 3957-3961.

(11) Endo, A. *J. Antibiot.* **1979**, *32*, 852-854.

(12) Slater, E. E.; MacDonald, J. S. *Drugs* **1988**, *36 Suppl 3*, 72-82.

(13) Biabani, M. A. F.; Laatsch, H. *J. Prakt. Chem.* **1998**, *340*, 589-607.

(14) Kohlmeyer, J.; Kohlmeyer, E. *Marine Mycology: The Higher Fungi*; Academic Press: London, 1979.

(15) Hyde, K. D.; Sarma, V. V.; Jones, E. B. G. *Morphology and Taxonomy of Higher Marine Fungi*. In *Marine Mycology: A Practical Approach*; Hyde, K. D., Pointing, S. B., Eds.; Fungal Diversity Press: Hong Kong, 2000; pp 172-204.

(16) Verbist, J. F.; Sallenave, C.; Pouchus, Y. F. *Marine Fungal Substances*. In *Studies in Natural Products Chemistry;* Atta-ur-Rahman Ed.; Elsevier: Amsterdam, 2000; Vol. 24, pp 979-1092.

(17) Bernan, V. S.; Greenstein, M.; Maiese, W. M. *Marine Microorganisms as a source of New Natural Products*. In *Advances In Applied Microbiology;* Neidleman, S. L., Laskin, A. I., Eds.; Academic: San Diego, 1997; Vol. 43, pp 57-90.

(18) Kobayashi, M.; Uehara, H.; Matsunami, K.; Aoki, S.; Kitagawa, I. *Tetrahedron Lett.* **1993**, *34*, 7925-7928.

(19) Abrell, L. M.; Borgeson, B.; Crews, P. *Tetrahedron Lett.* **1996**, *37*, 2331-2334.

(20) Cuomo, V.; Palomba, I.; Perretti, A.; Guerriero, A.; D'Ambrosio, M.; Pietra, F. *J. Mar. Biotechnol.* **1995**, *2*, 199-204.

(21) Burton, H. S.; Abraham, E. P. *Biochem. J.* **1951**, *50*, 16874.

(22) Jensen, P. R.; Fenical, W. *Marine microorganisms and drug discovery: current status and future potential,* In *Drugs from the Sea*; Fusetani, N., Ed.; Basel: Karger, 2000; pp 6-29.

(23) Kelecom, A. *An. Acad. Bras. Cienc.* **2002**, *74*, 151-170.

(24) Liberra, K.; Lindequist, U. *Pharmazie* **1995**, *50*, 583-588.

(25) Pietra, F. *Nat. Prod. Rep.* **1997**, *14*, 453-464.

(26) Belofsky, G. N.; Anguera, M.; Jensen, P. R.; Fenical, W.; Kock, M.

Chem. - Eur. J. **2000**, *6*, 1355-1360.

(27) Afiyatullov, S. S.; Kalinovsky, A. I.; Kuznetsova, T. A.; Isakov, V. V.; Pivkin, M. V.; Dmitrenok, P. S.; Elyakov, G. B. *J. Nat. Prod.* **2002**, *65*, 641-644.

(28) Afiyatullov, S. S.; Kuznetsova, T. A.; Isakov, V. V.; Pivkin, M. V.; Prokof'eva, N. G.; Elyakov, G. B. *J. Nat. Prod.* **2000**, *63*, 848-850.

(29) Isaka, M.; Suyarnsestakorn, C.; Tanticharoen, M.; Kongsaeree, P.; Thebtaranonth, Y. *J. Org. Chem.* **2002**, *67*, 1561-1566.

(30) Osterhage, C.; Kaminsky, R.; Koenig, G. M.; Wright, A. D. *J. Org. Chem.* **2000**, *65*, 6412-6417.

(31) Yamada, K.; Doi, M.; Yamada, T.; Minoura, K.; Numata, A. *Tennen Yuki Kagobutsu Toronkai Koen Yoshishu* **2000**, *42*, 397-402.

(32) Son, B. W.; Choi, J. S.; Kim, J. C.; Nam, K. W.; Kim, D. S.; Chung, H. Y.; Kang, J. S.; Choi, H. D. *J. Nat. Prod.* **2002**, *65*, 794-795.

(33) Fenical, W.; Jensen, P. R.; Cheng, X. C. *U.S.*; (University of California, San Diego, USA). Us 6066635, 2000, 6 pp.

(34) Jadulco, R.; Proksch, P.; Wray, V.; Sudarsono; Berg, A.; Graefe, U. *J. Nat. Prod.* **2001**, *64*, 527-530.

(35) Jadulco, R.; Brauers, G.; Edrada, R. A.; Ebel, R.; Wray, V.; Sudarsono; Proksch, P. *J. Nat. Prod.* **2002**, *65*, 730-733.

(36) Osterhage, C.; Koenig, G. M.; Hoeller, U.; Wright, A. D. *J. Nat. Prod.* **2002**, *65*, 306-313.

(37) Malmstrom, J.; Christophersen, C.; Barrero, A. F.; Oltra, J. E.; Justicia, J.; Rosales, A. *J. Nat. Prod.* **2002**, *65*, 364-367.

(38) Lin, Y.; Li, H.; Jiang, G.; Zhou, S.; Vrijmoed, L. L. P.; Jones, E. B. G. *Indian J. Chem.* **2002**, *41B*, 1542-1544.

(39) Renner, M. K.; Jensen, P. R.; Fenical, W. *J. Org. Chem.* **2000**, *65*, 4843-4852.

(40) Cueto, M.; Jensen, P. R.; Fenical, W. *Phytochemistry* **2000**, *55*, 223-226.

(41) Belofsky, G. N.; Jensen, P. R.; Fenical, W. *Tetrahedron Lett.* **1999**, *40*, 2913-2916.

(42) Omura, S.; Tomoda, H.; Tabata, N.; Ohyama, Y.; Abe, T.; Namikoshi, M. *J. Antibiot.* **1999**, *52*, 586-589.

(43) Chinworrungsee, M.; Kittakoop, P.; Isaka, M.; Rungrod, A.; Tanticharoen, M.; Thebtaranonth, Y. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1965-1969.

(44) Yamada, T.; Iwamoto, C.; Yamagaki, N.; Yamanouchi, T.; Minoura, K.; Yamori, T.; Uehara, Y.; Andoh, T.; Umemura, K.; Numata, A. *Tetrahedron* **2002**, *58*, 479-487.

(45) Namikoshi, M.; Akano, K.; Meguro, S.; Kasuga, I.; Mine, Y.; Takahashi, T.; Kobayashi, H. *J. Nat. Prod.* **2001**, *64*, 396-398.

(46) Namikoshi, M.; Kobayashi, H.; Yoshimoto, T.; Meguro, S.; Akano, K. *Chem. Pharm. Bull.* **2000**, *48*, 1452-1457.

(47) Son, B. W.; Kim, J. C.; Choi, H. D.; Kang, J. S. *Arch. Pharm. Res.* **2002**, *25*, 77-79.

(48) Iwamoto, C.; Yamada, T.; Ito, Y.; Minoura, K.; Numata, A. *Tetrahedron* **2001**, *57*, 2997-3004.

(49) Iwamoto, C.; Yamada, T.; Enomoto, S.; Minoura, K.; Numata, A. *Tennen Yuki Kagobutsu Toronkai Koen Yoshishu* **1999**, *41*, 601-606.

(50) Komatsu, K.; Shigemori, H.; Mikami, Y.; Kobayashi, J. *J. Nat. Prod.* **2000**, *63*, 408-409.

(51) Perpelescu, M.; Kobayashi, J.; Furuta, M.; Ito, Y.; Izuta, S.; Takemura, M.; Suzuki, M.; Yoshida, S. *Biochemistry* **2002**, *41*, 7610-7616.

(52) Byeng, W. S.; Jensen, P. R.; Kauffman, C. A.; Fenical, W. *Nat. Prod. Lett.* **1999**, *13*, 213-222.

(53) Cueto, M.; Jensen, P. R.; Kauffman, C.; Fenical, W.; Lobkovsky, E.; Clardy, J. *J. Nat. Prod.* **2001**, *64*, 1444-1446.

(54) Fenical, W.; Jensen, P. R.; Rowley, D. C. *PCT Int. Appl.*; (The Regents of the University of California, USA). Wo 0035943, 2000, 58 pp.

(55) Holler, U.; Konig, G.; Wright, A. D. *Eur. J. Org. Chem.* **1999**, 2949-2955.

(56) Uchida, R.; Tomoda, H.; Arai, M.; Omura, S. *J. Antibiot.* **2001**, *54*, 882-889.

(57) Komatsu, K.; Shigemori, H.; Kobayashi, J. *J. Org. Chem.* **2001**, *66*, 6189-6192.

(58) McDonald, L. A.; Abbanat, D. R.; Barbieri, L. R.; Bernan, V. S.; Discafani, C. M.; Greenstein, M.; Janota, K.; Korshalla, J. D.; Lassota, P.; Tischler, M.; Carter, G. T. *Tetrahedron Lett.* **1999**, *40*, 2489-2492.

(59) Lin, Y.; Wu, X.; Feng, S.; Jiang, G.; Luo, J.; Zhou, S.; Vrijmoed, L. L.; Jones, E. B.; Krohn, K.; Steingrover, K.; Zsila, F. *J. Org. Chem.* **2001**, *66*, 6252-6256.

(60) Poch, G. K.; Gloer, J. B. *Tetrahedron Lett.* **1989**, *30*, 3483-3486.

(61) Varoglu, M.; Corbett, T. H.; Valeriote, F. A.; Crews, P. *J. Org. Chem.* **1997**, *62*, 7078-7079.

(62) Shigemori, H.; Wakuri, S.; Yazawa, K.; Nakamura, T.; Sasaki, T.; Kobayashi, J. *Tetrahedron* **1991**, *47*, 8529-8534.

(63) Yamaguchi, K.; Tsuji, T.; Wakuri, S.; Yazawa, K.; Kondo, K.; Shigemori, H.; Kobayashi, J. *Biosci., Biotechnol., Biochem.* **1993**, *57*, 195-199.

(64) Takahashi, C.; Numata, A.; Ito, Y.; Matsumura, E.; Araki, H.; Iwaki, H.; Kushida, K. *J. Chem. Soc., Perkin Trans. 1* **1994**, 1859-1864.

(65) Takahashi, C.; Takai, Y.; Kimura, Y.; Numata, A.; Shigematsu, N.; Tanaka, H. *Phytochemistry* **1995**, *38*, 155-158.

(66) Takahashi, C.; Numata, A.; Matsumura, E.; Minoura, K.; Eto, H.; Shingu, T.; Ito, T.; Hasegawa, T. *J. Antibiot.* **1994**, *47*, 1242-1249.

(67) Takahashi, C.; Minoura, K.; Yamada, T.; Numata, A.; Kushida, K.; Shingu, T.; Hagishita, S.; Nakai, H.; Sato, T.; Harada, H. *Tetrahedron* **1995**, *51*, 3483-3498.

(68) Cui, C.-B.; Kakeya, H.; Okada, G.; Onose, R.; Ubukata, M.; Takahashi, I.; Isono, K.; Osada, H. *J. Antibiot.* **1995**, *48*, 1382-1384.

(69) Numata, A.; Takahashi, C.; Ito, Y.; Minoura, K.; Yamada, T.; Matsuda, C.; Nomoto, K. *J. Chem. Soc., Perkin Trans. 1* **1996**, 239-245.

(70) Takahashi, C.; Numata, A.; Yamada, T.; Minoura, K.; Enomoto, S.; Konishi, K.; Nakai, M.; Matsuda, C.; Nomoto, K. *Tetrahedron Lett.* **1996**, *37*, 655-658.

(71) Iwamoto, C.; Minoura, K.; Hagishita, S.; Nomoto, K.; Numata, A. *J. Chem. Soc., Perkin Trans. 1* **1998**, 449-456.

(72) Schlingmann, G.; Lisa, M.; Williams, D. R.; Carter, G. T. *J. Antibiot.* **1998**, *51*, 303-316.

(73) Albaugh, D.; Albert, G.; Bradford, P.; Cotter, V.; Froyd, J.; Gaughran, J.; Kirsch, D. R.; Lai, M.; Rehnig, A.; Sieverding, E.; Silverman, S. *J. Antibiot.* **1998**, *51*, 317-322.

(74) Renner, M. K.; Jensen, P. R.; Fenical, W. *J. Org. Chem.* **1998**, *63*, 8346-8354.

(75) Laatsch, H.; *Antibase- A Database for Rapid Identification of Microbial Natural Products*, Wiley-VCH Verlag Berlin GmbH, 2000.

(76) Arx, J. A. v.; Guarro, J.; Figueras, M. J. *The Ascomycete Genus Chaetomium*; Gebruder Borntraeger: Berlin, 1986.

(77) Tanenbaum, S. W. *Cytochalasins. Biochemical and Cell Biological Aspects;* North-Holland: Amsterdam, 1978.

(78) Natori, S.; Yahara, I. *Cytochalasins*. In *Mycotoxins and Phytoalexins;* Sharma R. P., Salunkhe, D. K., Eds.; CRC: Boca Raton, 1991; pp 291-336.

(79) Sekita, S.; Yoshihira, K.; Natori, S.; Kuwano, H. *Chem. Pharm. Bull.* **1982**, *30*, 1618-1628.

(80) Sekita, S.; Yoshihira, K.; Natori, S.; Kuwano, H. *Chem. Pharm. Bull.* **1982**, *30*, 1629-1638.

(81) Sekita, S.; Yoshihira, K.; Natori, S.; Udagawa, S.; Sakabe, F.; Kurata, H.; Umeda, M. *Chem. Pharm. Bull.* **1982**, *30*, 1609-1617.

(82) Ichihara, A.; Katayama, K.; Teshima, H.; Oikawa, H.; Sakamura, S. *Biosci. Biotech. Biochem.* **1996**, *60*, 360-361.

(83) Ko, H.-R.; Kim, B. Y.; Ahn, S.-C.; Oh, W. K.; Kim, J.-H.; Lee, H. S.; Kim, H.-M.; Han, S.-B.; Mheen, T.-I.; Ahn, J.-S. *J. Microbiol. Biotechnol.* **1998**, *8*, 705-709.

(84) Namgoong, J.; Yeon, S.-W.; Paek, N.-S.; Kim, T.-H.; Kim, Y.-H.; Kim, C.-J.; Kim, K.-W. *Sanop Misaengmul Hakhoechi* **1998**, *26*, 137-142.

(85) Kang, J. G.; Kim, K. K.; Kang, K. Y. *Agric. Chem. Biotechnol. (Engl. Ed.)* **1999**, *42*, 146-150.

(86) Safe, S.; Taylor, A. *J. Chem. Soc., Perkin Trans. 1* **1972**, 472-479.

(87) Kikuchi, T.; Kadota, S.; Nakamura, K.; Nishi, A.; Taga, T.; Kaji, T.; Osaki, K.; Tubaki, K. *Chem. Pharm. Bull.* **1982**, *30*, 3846-3848.

(88) Saito, T.; Suzuki, Y.; Koyama, K.; Natori, S.; Iitaka, Y.; Kinoshita, T. *Chem. Pharm. Bull.* **1988**, *36*, 1942-1956.

(89) Saito, T.; Koyama, K.; Natori, S.; Iitaka, Y. *Tetrahedron Lett.* **1985**, *26*, 4731-4734.

(90) Jen, W. C.; Jones, G. A. *Can. J. Microbiol.* **1983**, *29*, 1399-1404.

(91) Di-Pietro, A.; Gut Rella, M.; Pachlatko, J. P.; Schwinn, F. J. *Phytopathology* **1992,** *82,* 131-135.

(92) Brewer, D.; Hannah, D. E.; Taylor, A. *Can. J. Microbiol.* **1966,** *12*, 1187-1195.

(93) Sekita, S.; Yoshihira, K.; Natori, S. *Chem. Pharm. Bull.* **1980**, *28*, 2428-2435.

(94) Koyama, K.; Natori, S. *Chem. Pharm. Bull.* **1987**, *35*, 578-584.

(95) Koyama, K.; Ominato, K.; Natori, S.; Tashiro, T.; Tsuruo, T. *J. Pharmacobio-Dyn.* **1988**, *11*, 630-635.

(96) Bills, G. F.; Lingham, R. B.; Shafiee, A.; Silverman, K. C.; Singh, S. B.; Zink, D. L.; Pelaez, F.; Teran, A. M. *PCT Int. Appl.*; (Merck & Co., Inc., USA). Wo 9834932, 1998, 46 pp.

(97) Brewer, D.; Jerram, W. A.; Taylor, A. *Can. J. Microbiol.* **1968**, *14*, 861-866.

(98) Sekita, S. *Chem. Pharm. Bull.* **1983**, *31*, 2998-3001.

(99) Brewer, D.; Jerram, W. A.; Meiler, D.; Taylor, A. *Can. J. Microbiol.* **1970**, *16*, 433-440.

(100) Brewer, D.; Maass, W. S. G.; Taylor, A. *Can. J. Microbiol.* **1977**, *23*, 845-851.

(101) Brewer, D.; Jen, W. C.; Jones, G. A.; Taylor, A. *Can. J. Microbiol.* **1984**, *30*, 1068-1072.

(102) Brewer, D.; Taylor, A. *Can. J. Microbiol.* **1978**, *24*, 1082-1086.

(103) Brewer, D.; McInnes, A. G.; Smith, D. G.; Taylor, A.; Walter, J. A.; Loosli, H. R.; Kis, Z. L. *J. Chem. Soc., Perkin Trans. 1* **1978**, 1248-1251.

(104) Waksman, S. A.; Bugie, E. *J. Bact.* **1944**, *48*, 527-530.

(105) Brewer, D.; Duncan, J. M.; Jerram, W. A.; Leach, C. K.; Safe, S.; Taylor, A.; Vining, L. C.; Archibald, R. M.; Stevenson, R. G.; *et al. Can. J. Microbiol.* **1972**, *18*, 1129-1137.

(106) Trown, P. W. *Biochem. Biophys. Res. Commun.* **1968**, *33*, 402-407.

(107) Pitt, J. I.; Hocking, A. D. *Fungi and Food Spoilage*, 2nd ed.; Blackie Academic & Professional: London, 1997.

(108) Sekita, S.; Yoshihira, K.; Natori, S.; Udagawa, S.; Sakabe, F.; Kurata, H. *Maikotokishin (Tokyo)* **1982**, *14*, 24-25.

(109) Sekita, S.; Yoshihira, K.; Natori, S. *Chem. Pharm. Bull.* **1983**, *31*, 490-498.

(110) Oikawa, H.; Murakami, Y.; Ichihara, A. *J. Chem. Soc., Perkin Trans. 1* **1992**, 2949-2954.

(111) Oikawa, H.; Murakami, Y.; Ichihara, A. *J. Chem. Soc., Perkin Trans. 1* **1992**, 2955-2959.

(112) Izawa, Y.; Hirose, T.; Shimizu, T.; Koyama, K.; Natori, S. *Tetrahedron* **1989**, *45*, 2323-2335.

(113) Takahashi, M.; Koyama, K.; Natori, S. *Chem. Pharm. Bull.* **1990**, *38*, 625-628.

(114) Yaoita, Y.; Amemiya, K.; Ohnuma, H.; Furumura, K.; Masaki, A.; Matsuki, T.; Kikuchi, M. *Chem. Pharm. Bull.* **1998**, *46*, 944-950.

(115) Iguchi, K.; Shimura, H.; Yang, Z.; Yamada, Y. *Steroids* **1993**, *58*, 410-413.

(116) Kim, S.-W.; Park, S.-S.; Min, T.-J.; Yu, K.-H. *Bull. Korean Chem. Soc.* **1999**, *20*, 819-823.

(117) Gonzalez, A. G.; Barrera, J. B.; Toledo Marante, F. J. *Phytochemistry* **1983**, *22*, 1049-1050.

(118) Kahlos, K.; Kangas, L.; Hiltunen, R. *Planta Med.* **1989**, *55*, 389-390.

(119) Gunatilaka, A. A. L.; Gopichand, Y.; Schmitz, F. J.; Djerassi, C. *J. Org. Chem.* **1981**, *46*, 3860-3866.

(120) Lindequist, U.; Lesnau, A.; Teuscher, E.; Pilgrim, H. *Pharmazie* **1989**, *44*, 579-580.

(121) Kim, D. S.; Baek, N.-I.; Oh, S. R.; Jung, K. Y.; Lee, I. S.; Kim, J. H.; Lee, H.-K. *Arch. Pharm. Res.* **1997**, *20*, 201-205.

(122) Sutton, B. C. *The Coelomycetes: Fungi Imperfecti with Pycnidia, Acervuli and Stromata*; Commonwealth Mycological Institute: Kew, Surrey, England, 1980.

(123) de Gruyter, J.; Scheer, P. *Journal of Phytopathology-Phytopathologische Zeitschrift* **1998**, *146*, 411-415.

(124) Punithalingham-E; Harling, R. *Mycol. Res.* **1993**, *9,* 1299-1304.

(125) Sugano, M.; Sato, A.; Iijima, Y.; Oshima, T.; Furuya, K.; Kuwano, H.; Hata, T.; Hanzawa, H. *J. Am. Chem. Soc.* **1991**, *113*, 5463-5464.

(126) Sugano, M.; Sato, A.; Iijima, Y.; Furuya, K.; Haruyama, H.; Yoda, K.; Hata, T. *J. Org. Chem.* **1994**, *59*, 564-569.

(127) Sugano, M.; Sato, A.; Iijima, Y.; Furuya, K.; Kuwano, H.; Hata, T. *J. Antibiot.* **1995**, *48*, 1188-1190.

(128) Sato, A.; Sugano, M.; Oshima, T.; Haruyama, H.; Furuya, K. *Jpn. Kokai Tokkyo Koho*; (Sankyo Co., Ltd., Japan). Jp 03216197, 1991, 7 pp.

(129) Evidente, A.; Lanzetta, R.; Capasso, R.; Andolfi, A.; Bottalico, A.; Vurro, M.; Zonno, M. C. *Phytochemistry* **1995**, *40*, 1637-1641.

(130) Evidente, A.; Lanzetta, R.; Capasso, R.; Andolfi, A.; Vurro, M.; Zonno, M. C. *Phytochemistry* **1997**, *44*, 1041-1045.

(131) Evidente, A.; Capasso, R.; Andolfi, A.; Vurro, M.; Zonno, M. C.

Phytochemistry **1998**, *48*, 941-945.

(132) Capasso, R.; Evidente, A.; Vurro, M. *Phytochemistry* **1991**, *30*, 3945-3950.

(133) Evidente, A.; Lanzetta, R.; Capasso, R.; Vurro, M.; Bottalico, A. *Tetrahedron* **1992**, *48*, 6317-6324.

(134) Evidente, A.; Capasso, R.; Vurro, M.; Bottalico, A. *Nat. Toxins* **1996**, *4*, 53-57.

(135) Griffiths, L. A. *J. Exptl. Botany* **1959**, *10*, 437-442.

(136) Bassett, E. W.; Tanenbaum, S. W. *Experientia* **1958**, *14*, 38-40.

(137) Esaki, H.; Onozaki, H.; Osawa, T.; Kawakishi, S. *J. Agric. Food Chem.* **1997**, *45*, 2020-2024.

(138) Chen, C.-H.; Shaw, C.-Y.; Chen, C.-C.; Tsai, Y.-C. *J. Nat. Prod.* **2002**, *65*, 740-741.

(139) Engel, B. G.; Brzeski, W. *Helv. Chim. Acta* **1947**, *30*, 1472-1478.

(140) Sequin-Frey, M.; Tamm, C. *Helv. Chim. Acta* **1971**, *54*, 851-861.

(141) Makino, M.; Endoh, T.; Ogawa, Y.; Watanabe, K.; Fujimoto, Y. *Heterocycles* **1998**, *48*, 1931-1934.

(142) Ishikawa, Y.; Morimoto, K.; Hamasaki, T. *Yukagaku* **1986**, *35*, 903-906.

(143) Booth, C. *The Genus Fusarium*; Commonwealth Agricultural Bureaux: Bucks, England, 1971.

(144) Bamburg, J. R. *Adv. Chem. Ser.* **1976**, *149*, 144-162.

(145) Ueno, Y. *Ann. Nutr. Aliment.* **1977**, *31*, 885-900.

(146) Ueno, Y.; Sato, N.; Ishii, K.; Sakai, K.; Enomoto, M. *Jap. J. Exp. Med.* **1972**, *42*, 461-472.

(147) Li, F.-Q.; Luo, X.-Y.; Yoshizawa, T. *Nat. Toxins* **1999**, *7*, 93-97.

(148) Bannwart, G.; Reyes, F. G. R. *Revista Brasileira de Toxicologia* **2001**, *14*, 37-51.

(149) Ueno, Y. *Dev. Food Sci.* **1983**, *4*, 135-146.

(150) McLean, M. *Mycopathologia* **1996**, *133*, 163-179.

(151) Eudes, F.; Comeau, A.; Rioux, S.; Collin, J. *Can. J. Plant Pathol.* **2000**, *22*, 286-292.

(152) Isaka, M.; Punya, J.; Lertwerawat, Y.; Tanticharoen, M.; Thebtaranonth, Y. *J. Nat. Prod.* **1999**, *62*, 329-331.

(153) Mekhancha-Dahel, C.; Lafarge-Frayssinet, C.; Venuat, A. M.; Rosenfeld, C.; Frayssinet, C. *J. Cell. Pharmacol.* **1992**, *2*, 343-350.

(154) Bilgrami, K. S.; Sahay, S. S.; Shrivastava, A. K.; Rahman, M. F. *Indian Phytopathol.* **1992**, *45*, 469-471.

(155) Sugiura, Y. *Mycotoxins* **2000**, *50*, 125-128.

(156) Gaumy, J. L.; Bailly, J. D.; Benard, G.; Guerre, P. *Revue de Medecine Veterinaire (Toulouse)* **2001**, *152*, 123-136.

(157) Mirocha, C. J.; Christensen, C. M.; Nelson, G. H. *Microbial Toxins* **1971**, *7*, 107-138.

(158) Doko, M. B.; Canet, C.; Brown, N.; Sydenham, E. W.; Mpuchane, S.; Siame, B. A. *J. Agric. Food Chem.* **1996**, *44*, 3240-3243.

(159) Sweeney, M. J.; Dobson, A. D. W. *Int. J. Food Microbiol.* **1998**, *43*, 141-158.

(160) Gelderblom, W. C. A.; Jaskiewicz, K.; Marasas, W. F. O.; Thiel, P. G.; Horak, R. M.; Vleggaar, R.; Kriek, N. P. J. *Appl. Environ. Microbiol.* **1988**, *54*, 1806-1811.

(161) Branham, B. E.; Plattner, R. D. *J. Nat. Prod.* **1993**, *56*, 1630-1633.

(162) Musser, S. M.; Gay, M. L.; Mazzola, E. P.; Plattner, R. D. *J. Nat. Prod.*

1996, *59*, 970-972.

(163) Mallmann, C. A.; Santurio, J. M.; Dilkin, P. *Revista de Microbiologia* **1999**, *30*, 249-252.

(164) Haschek, W. M.; Gumprecht, L. A.; Smith, G.; Tumbleson, M. E.; Constable, P. D. *Environ. Health Perspect.* **2001**, *109*, 251-257.

(165) Marasas, W. F. O. *Environ. Health Perspect.* **2001**, *109 Supple.*, 239-243.

(166) Qiu, M.; Liu, X.; Wang, Y.; Zhang, C. *Weisheng Yanjiu* **2001**, *30*, 365-366.

(167) Ueno, Y.; Iijima, K.; Wangi, S. D.; Sugiura, Y.; Sekijima, M.; Tanaka, T.; Chen, C.; Yu, S. Z. *Food Chem. Toxicol.* **1997**, *35*, 1143-1150.

(168) Gelderblom, W. C. A.; Snyman, S. D.; Abel, S.; Lebepe-Mazur, S.; Smuts, C. M.; Van der Westhuizen, L.; Marasas, W. F. O.; Victor, T. C.; Knasmuller, S.; Huber, W. *Adv. Exp. Med. Biol.* **1996**, *392*, 279-296.

(169) Merrill, A. H., Jr.; Wang, E.; Vales, T. R.; Smith, E. R.; Schroeder, J. J.; Menaldino, D. S.; Alexander, C.; Crane, H. M.; Xia, J.; *et al.* *Adv. Exp. Med. Biol.* **1996**, *392*, 297-306.

(170) Riley, R. T.; Norred, W. P.; Voss, K. A.; Sharma, R. P.; Merrill, A. H. Jr. *Kinki Daigaku Nogaku Sogo Kenkyusho Hokoku* **2000**, *8*, 55-65.

(171) Shibata, S.; Morishita, E.; Takeda, T.; Sakata, K. *Tetrahedron Lett.* **1966**, 4855-4860.

(172) Arsenault, G. P. *Tetrahedron Lett.* **1965**, 4033-4037.

(173) Cornforth, J. W.; Ryback, G.; Robinson, P. M.; Park, D. *J. Chem. Soc. C* **1971**, 2786-2788.

(174) Kern, H.; Naef-Roth, S. *Phytopathol. Z.* **1965**, *53*, 45-64.

(175) Arsenault, G. P. *Tetrahedron* **1968**, *24*, 4745-4749.

(176) Vesonder, R. F.; Golinski, P. *Metabolites of Fusarium*. In *Fusarium:*

Mycotoxins, Taxonomy and Pathogenicity, 1st ed.; Chelkowski, J., Ed.; Elsevier: Amsterdam, 1989; Vol. 2, pp 1-39.

(177) Kjaer, D.; Kjaer, A.; Pedersen, C.; Bu'lock, J. D.; Smith, J. R. *J. Chem. Soc., Perkin Trans. 1* **1971**, *16*, 2792-2797.

(178) de Boer, J. J.; Bright, D.; Dallinga, G.; Hewitt, T. G. *J. Chem. Soc., Perkin Trans. 1* **1971**, *16*, 2788-2791.

(179) Chelkowski, J.; Zajkowski, P.; Visconti, A. *Mycotoxin Res.* **1992**, *8*, 73-76.

(180) Kreitman, G.; Nord, F. F. *Arch. Biochem.* **1949**, *21*, 457-458.

(181) Brewer, D.; Arsenault, G. P.; Wright, J. L. C.; Vining, L. C. *J. Antibiot.* **1973**, *26*, 778-781.

(182) Terashima, N.; Ishida, M.; Hamasaki, T.; Hatsuda, Y. *Phytochemistry* **1972**, *11*, 2880.

(183) Fuska, J.; Proksa, B.; Fuskova, A. *Neoplasma* **1975**, *22*, 335-338.

(184) Balan, J.; Fuska, J.; Kuhr, I.; Kuhrova, V. *Folia Microbiol. (Prague)* **1970**, *15*, 479-484.

(185) Kovac, L.; Bohmerova, E.; Fuska, J. *J. Antibiot.* **1978**, *31*, 616-620.

(186) Kagamizono, T.; Nishino, E.; Matsumoto, K.; Kawashima, A.; Kishimoto, M.; Sakai, N.; He, B.-M.; Chen, Z.-X.; Adachi, T.; *et al.* *J. Antibiot.* **1995**, *48*, 1407-1412.

(187) Hamill, R. L.; Higgens, C. E.; Boaz, H. E.; Gorman, M. *Tetrahedron Lett.* **1969**, *49*, 4255-4258.

(188) Gupta, S.; Montllor, C.; Hwang, Y.-S. *J. Nat. Prod.* **1995**, *58*, 733-738.

(189) Gupta, S.; Krasnoff, S. B.; Underwood, N. L.; Renwick, J. A. A.; Roberts, D. W. *Mycopathologia* **1991**, *115*, 185-189.

(190) Mao, H. *Zhongcaoyao* **1985**, *16*, 293-294.

(191) Bernardini, M.; Carilli, A.; Pacioni, G.; Santurbano, B. *Phytochemistry* **1975**, *14*, 1865.

(192) Nilanonta, C.; Isaka, M.; Kittakoop, P.; Palittapongarnpim, P.; Kamchonwongpaisan, S.; Pittayakhajonwut, D.; Tanticharoen, M.; Thebtaranonth, Y. *Planta Med.* **2000**, *66*, 756-758.

(193) Ovchinnikov, Y. A.; Ivanov, V. T.; Mikhaleva, I. I. *Tetrahedron Lett.* **1971**, 159-162.

(194) Samson, R. A. *Taxonomy - Current Concepts of Aspergillus Systematics*, In *Aspergillus*; Smith, J. E., Ed.; Plenum Press: New York, 1994; pp 1-22.

(195) Pitt, J. I.; Hocking, A. D. *Fungi and Food Spoilage*, 1. Edition; Academic Press, 1985.

(196) Conning, D. M. *Systemic Toxicity due to Food Stuffs*. In *Toxic Hazards in Food*; Conning, D. M., Lansdown, A. B. G., Eds.; Groom Helm: London, 1983; pp 5-22.

(197) Stoloff, L.; Van Egmond, H. P.; Park, D. L. *Food Addit. Contam.* **1991**, *8*, 213-222.

(198) Anon *Br. Med. J.* **1962**, *2*, 172-174.

(199) Robens, J. F.; Richard, J. L. *Rev. Environ. Contam. Toxicol.* **1992**, *127*, 69-94.

(200) Nesheim, S. *J. Ass. Offic. Anal. Chem.* **1969**, *52*, 975-979.

(201) Pohland, A. E.; Nesheim, S.; Friedman, L. *Pure Appl. Chem.* **1992**, *64*, 1029-1046.

(202) Doster, R. C.; Sinnhuber, R. O.; Pawlowski, N. E. *Food Cosmet. Toxicol.* **1974**, *12*, 499-505.

(203) Xiao, H.; Madhyastha, S.; Marquardt, R. R.; Li, S.; Vodela, J. K.; Frohlich, A. A.; Kemppainen, B. W. *Toxicol. Appl. Pharmacol.* **1996**, *137*, 182-192.

(204) Di Paolo, N.; Guarnieri, A.; Garosi, G.; Sacchi, G.; Mangiarotti, A. M.; Di Paolo, M. *Nephrology, Dialysis, Transplantation* **1994**, *9 Suppl 4*, 116-120.

(205) Bach, P. H.; Gregg, N. J.; Delacruz, L. *Food Chem. Toxicol.* **1992**, *30*, 205-211.

(206) Dirheimer, G.; Creppy, E. E.; Roeschenthaler, R. *Hommage Professeur Rene Truhaut* **1984**, 301-305.

(207) Cox, R. H.; Cole, R. J. *J. Org. Chem.* **1977**, *42*, 112-114.

(208) Terao, K. *J. Toxicol., Toxin Rev.* **1983**, *2*, 77-110.

(209) Bradner, W. T.; Bush, J. A.; Myllymaki, R. W.; Nettleton, D. E. Jr.; O'Herron, F. A. *Antimicrob. Agents Chemother.* **1975**, *8*, 159-163.

(210) Yamazaki, M.; Fujimoto, H.; Kawasaki, T. *Chem. Pharm. Bull.* **1980**, *28*, 245-254.

(211) Yamazaki, M.; Fujimoto, H.; Maebayashi, Y.; Okuyama, E. *Tennen Yuki Kagobutsu Toronkai Koen Yoshishu* **1978**, *21*, 14-21.

(212) Yamazaki, M.; Fujimoto, H.; Okuyama, E. *Tetrahedron Lett.* **1976**, 2861-2864.

(213) Horie, Y.; Yamazaki, M. *Nippon Kingakkai Kaiho* **1981**, *22*, 113-119.

(214) Yamazaki, M.; Fujimoto, H.; Kawasaki, T. *Tetrahedron Lett.* **1975**, 1241-1244.

(215) Yamazaki, M.; Sasago, K.; Miyaki, K. *J. Chem. Soc., Chem. Commun.* **1974**, 408-409.

(216) Yamazaki, M.; Suzuki, S.; Miyaki, K. *Chem. Pharm. Bull.* **1971**, *19*, 1739-1740.

(217) Yamazaki, M.; Suzuki, S.; Kukita, K. *J. Pharmacobio-Dyn.* **1979**, *2*, 119-125.

(218) Yamazaki, M.; Suzuki, S. *Dev. Toxicol. Environ. Sci.* **1986**, *12*, 273-282.

(219) Rabindran, S. K.; He, H.; Singh, M.; Brown, E.; Collins, K. I.; Annable, T.; Greenberger, L. M. *Cancer Res.* **1998**, *58*, 5850-5858.

(220) He, H.; Rabindran, S. G.; Greenberger, L. M.; Carter, G. T. *Med. Chem. Res.* **1999**, *9*, 424-437.

(221) van Loevezijn, A.; Allen, J. D.; Schinkel, A. H.; Koomen, G. J. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 29-32.

(222) VanMiddlesworth, F.; Giacobbe, R. A.; Lopez, M.; Garrity, G.; Bland, J. A.; Bartizal, K.; Fromtling, R. A.; Polishook, J.; Zweerink, M.; *et al. J. Antibiot.* **1992**, *45*, 861-867.

(223) Zweerink, M. M.; Edison, A. M.; Wells, G. B.; Pinto, W.; Lester, R. L. *J. Biol. Chem.* **1992**, *267*, 25032-25038.

(224) Kozakiewicz, Z. *Aspergillus Species on Stored Products*; C. A. B International: Oxon, 1989.

(225) Cable, K. M.; Herbert, R. B.; Knaggs, A. R.; Mann, J. *J. Chem. Soc., Perkin Trans. 1* **1991**, 595-599.

(226) Achenbach, H.; Strittmatter, H.; Kohl, W. *Chem. Ber.* **1972**, *105*, 3061-3066.

(227) Buechi, G.; Luk, K. C.; Kobbe, B.; Townsend, J. M. *J. Org. Chem.* **1977**, *42*, 244-246.

(228) Takatsuki, A.; Suzuki, S.; Ando, K.; Tamura, G.; Arima, K. *J. Antibiot.* **1968**, *21*, 671-675.

(229) Tsuruoka, T.; Fukuyasu, H.; Kawaharajo, K.; Koyama, M.; Kurihara, H. *Eur. Pat. Appl.*; (Meiji Seika Kaisha Ltd., Japan). Ep 486944, 1992, 5 pp.

(230) Cole, R. J.; Kirksey, J. W.; Dorner, J. W.; Bedell, D. M.; Springer, J. P.; Chexal, K. K.; Clardy, J. C.; Cox, R. H. *Agric. Food Chem.* **1977**, *25*, 826-830.

(231) Hino, T.; Kawate, T.; Nakagawa, M. *Tetrahedron* **1989**, *45*, 1941-1944.

(232) Cui, C.-B.; Kakeya, H.; Okada, G.; Onose, R.; Osada, H. *J. Antibiot.* **1996**, *49*, 527-533.

(233) Halasz, J.; Podanyi, B.; Vasvari-Debreczy, L.; Szabo, A.; Hajdu, F.; Bocskei, Z.; Hegedus-Vajda, J.; Gyorbiro, A.; Hermecz, I. *Tetrahedron* **2000**, *56*, 10081-10085.

(234) Maevskii, M. M.; Romanenko, E. A.; Mol'kov, Y. N.; Urazova, A. P.; Timofeevskaya, E. A.; Bondareva, A. S.; Mazaeva, V. G. *Tr. S'ezda Onkol. Ukr. SSR,* **1967**, 186-187.

(235) Stepien, H.; Grochal, M.; Zielinski, K. W.; Mucha, S.; Kunert-Radek, J.; Kulig, A.; Stawowy, A.; Pisarek, H. *J. Endocrinol.* **1996**, *150*, 99-106.

(236) Ingber, D.; Fujita, T.; Kishimoto, S.; Sudo, K.; Kanamaru, T.; Brem, H.; Folkman, J. *Nature (London)* **1990**, *348*, 555-557.

(237) Sin, N.; Meng, L.; Wang, M. Q. W.; Wen, J. J.; Bornmann, W. G.; Crews, C. M. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94*, 6099-6103.

(238) Dai, X. B.; Griffith, E. C.; Liu, J. O. *Book of Abstracts, 219th ACS National Meeting,* San Francisco, CA, March 26-30, 2000, MEDI-061.

(239) Conteas, C. N.; Berlin, O. G. W.; Ash, L. R.; Pruthi, J. S. *Am. J. Trop. Med. Hyg.* **2000**, *63*, 121-127.

(240) Molina, J.-M.; Goguel, J.; Sarfati, C.; Michiels, J.-F.; Desportes-Livage, I.; Balkan, S.; Chastang, C.; Cotte, L.; Maslo, C.; Struxiano, A.; Derouin, F.; Decazes, J.-M. *AIDS (London)* **2000**, *14*, 1341-1348.

(241) Girardeau, J. H., Jr. *Environ. Entomol.* **1972**, *1*, 519-520.

(242) *Jpn. Tokkyo Koho*; (Takeda Chemical Industries, Ltd., Japan). Jp 57043204, 1982, 5 pp.

(243) Speare, D. J.; Athanassopoulou, F.; Daley, J.; Sanchez, J. G. *J. Comp. Pathol.* **1999**, *121*, 241-248.

(244) McCowen, M. C.; Callender, M. E.; Lawlis, J. F. Jr. *Science* **1951**, *113*, 202-203.

(245) le Gouvello, R.; Pobel, T.; Richards, R. H.; Gould, C. *Aquaculture* **1999**, *171*, 27-40.

(246) Ellis, G. Department of Chemistry, University of Canterbury, 2002, personal communication.